1995

Characterization of genes expressed during vegetative organ development in potato (Solanum tuberosum L.)

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Characterization of genes expressed during vegetative organ development in potato (*Solanum tuberosum* L.)

by

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A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the degree of

DOCTOR OF PHILOSOPHY

Department: Horticulture
Interdepartmental Major: Plant Physiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Interdepartmental Major

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1995

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CHAPTER 1. GENERAL INTRODUCTION

The vascular plant consists of two systems: The shoot system consists of stems, leaves, and flowers; whereas the root system consists of primary roots and lateral roots. Organs are developed from meristem cells in the apices of each system. Genetic regulation, signal transduction by hormonal and environmental factors, and biochemical processes in cells and organelles are involved in the complex developmental pathways of plants. Currently, the molecular and genetic mechanisms involved in the processes of plant reproductive cell/tissue determination, differentiation, and development are being intensively investigated. However, the molecular mechanisms of vegetative organ development are poorly understood, because the developmental processes of indeterminate vegetative apical meristems are more complex and involve various types of differentiation and determination processes (Medford, 1992).

Potato Tuber Development

The cultivated potato (*Solanum tuberosum*) produces the vegetative storage organ known as a tuber which develops from modified underground stems called stolons (Cutter, 1982; Peterson et al., 1985; Part et al., 1990). *Solanum tuberosum* is a tetraploid obtained by chromosome doubling from a hybrid between two diploid species, *Solanum stentotomum* and *Solanum sparsipilum* (Ramanna and Hermsen, 1979). Potato tuberization is characterized by a morphological change in the growth of the stolon from extension growth to radial growth and the physiological change in accumulation of starch and tuber specific proteins (Park et al., 1985; Ewing, 1990). Tuberization is induced by environmental factors such as cool temperature, low levels of nitrogen, and short photoperiod (Ewing, 1990). In addition, the tuberization process is stimulated by physiological factors including ABA (Krauss and Marschner, 1982), cytokinins (Sattelmacher and Marschner., 1978), methyl jasmonic acid derivatives (Koda et al., 1988), and high sucrose concentrations (Wenzler et al., 1989; Perl et

al., 1991). Gibberellic acid acts as an inhibitor of tuberization (Krauss and Marschner, 1982; Hannapel et al., 1985).

Using antisense gene transformation, inhibition of granule-bound starch synthase (Visser et al., 1991), ADP-glucose pyrophophorylase (Mueller-Rober et al., 1992), UDP-glucose pyrophosphorylase (Zrenner et al., 1993), and patatin (Höfgen and Willmitzer, 1992) resulted in significant reduction in the levels of these proteins, but no changes were observed in tuber morphology, indicating that these genes are not directly involved in tuber morphogensis. Recently, Taylor et al. (1991; 1992a; 1992b) observed that transcript levels of an α-tubulin isotype gene, ribosomal protein S19 and L7 genes for cytosolic ribosomal components, and S-adenosylmethionine decarboxylase (SAMDC) gene were significantly increased in stolon tips during early tuberization. However, the function of these genes during tuberization has not been elucidated. Although much effort has focused on identifying genes involved in tuber morphogenesis, there is no information about genes involved directly in tuberization. To understand this developmental process, the characterization of genes expressed in early tuber development is necessary.

MADS-box Proteins and Transcription Factors

Gene expression involves the interaction of the regulatory region of the gene and complex transcriptional machinery including RNA polymerases and regulatory protein factors. Many DNA-binding proteins are transcription factors that mediate the selective expression of certain genes. Those transcription factors have been isolated and characterized in both prokaryotes (Hegde et al., 1992) and eukaryotes (Miller et al., 1985; Herr et al., 1988; Landschulz et al., 1988; Murre et al., 1989; Sommer et al., 1990). Transcription factors can up or down regulate gene transcription, resulting in cell development, differentiation, and cell growth (Kelly et al., 1983; Verma et al., 1986; Otting et al., 1990; Sommer et al., 1990). The

essential characteristics of transcription factors are the abilities to bind DNA in a sequence-specific manner, and interaction with other transcription factors or the RNA polymerases.

When homeotic genes are mutated, the organ structure(s) of one system are transformed into the corresponding organ structure(s) of another morphological system, leading to morphogenic mutants known as homeotic mutants. Many homeotic genes have been identified as being involved in flower development in numerous plant species (Schwarz-Sommer et al., 1990; Sommer et al., 1990; Yanofsky et al., 1990; Ma et al., 1991; Angenent et al., 1992; Huijser et al., 1992; Jack et al., 1992; Mandel et al., 1992b). Floral organ homeotic genes can be categorized in three classes based on their mutant phenotypes (reviewed in Weigel and Meyerowitz, 1994): 1) floral meristem identity genes involved in the formation of floral primodia such as *LFY* (Weigel et al., 1992), *AP1* (Mandel et al., 1992b), and *CAL* (Kempin et al., 1995) in *Arabidopsis* and *FLO* (Carpenter and Coen, 1990) in *Antirrhinum majus* ; 2) floral organ identity genes involved in the development of floral organs (sepals, petals, stamens, and carpels) such as *AP1* (Mandel et al., 1992b), *AP3* (Jack et al., 1992), and *AG* (Yanofsky et al., 1990) of *Arabidopsis* and *PLE* (Bradley et al., 1993), *SQUA* (Huijer et al., 1992), *DEF A* (Sommer et al., 1990), and *GLO* (Tröbner et al., 1992) of *Antirrhinum majus* ; and 3) cadastral genes such as *AG* (Yanofsky et al., 1990), *SUP*(Bowman et al., 1992), and *AP2* (Bowman et al., 1989) of *Arabidopsis* and *PLE* (Bradley et al., 1993) of *Antirrhinum majus*. The cadastral genes are involved in regulation of organ identity genes.

MADS-box genes are a family of floral homeotic genes. Examples include: *DEF A*, *SQUA*, *GLO*, and *PLE* of *Antirrhium*, *AG*, *AGLs* (Ma et al., 1991), *AP3*, *AP1*, and *CAL* of *Arabidopsis*; and *St-Def* (Gracia-Maroto et al., 1993) of *Solanum*. MADS-box proteins contain two conserved regions (Schwarz-Sommer, et al., 1990; Ma et al., 1991). One is the DNA-binding domain, designated the MADS-box domain (Schwarz-Sommer, et al., 1990). This region consists of 56 amino acid residues of conserved motifs and is present in transcription factors of MCM1 in yeast (Passmore et al., 1988), AG in *Arabidopsis*, DEF A in

Antirrhinum, and SRF (serum response factor) in human (Norman et al., 1988). DNA-protein binding analysis using ***in vitro*** footprinting, UV-cross linking, and gel-shift assays of MCM1, SRF, DEF A, and GLO(GLOBOSA) showed that MADS-box proteins bound DNA, and it was proposed that the MADS-box domain functions in DNA-binding (Norman et al., 1988; Schröter et al., 1990; Schwarz-Sommer et al., 1992; Tröbner et al., 1992). The transcription factor MCM1 of the yeast *Saccaromyces cerevisae* controls a number of different genes by specific DNA-binding with other transcription factors to regulate mating types (Passmore et al., 1988). Human serum response factor (SRF) is a transcriptional factor for many genes that are transiently activated by growth factor stimulation (Schröter et al., 1990). These four proteins showed very high sequence homology in the DNA-binding domain, binding the DNA sequence $CC(A/T)_6GG$ (termed CArG box by Minty and Kedes, 1986) on promoters of each target gene. Cañas et al. (1994) observed that petunia flower MADS-box protein FBP1, which is encoded by *fbp1* and required for the correct initiation and determination of petals and stamens of petunia, was localized in the nuclei of petal cells, supporting the hypothesis that plant MADS-box proteins function as transcription factors. The second conserved domain of MADS-box proteins is designated the K-box (Ma et al., 1991) because of its structural similarity to the coiled-coil domain of keratin (Tyner at al., 1985). It is known that only plant MADS-box proteins have K-box domains, whereas SRF of vertebrate and MCM1 of yeast do not (Ma et al., 1991). Schwarz-Sommer et al. (1992) proposed that the K-box domain mediates protein-protein interactions of the MADS-box proteins to form homo- or heterodimers that facilitate DNA-binding with other regulatory factors. However, little is known about the function of the K-box domain.

Early tuberization involves biological processes such as plastid differentiation, amyloplast development, carbon partitioning, starch accumulation, cell division, and the induction of specific proteins, resulting in morphological changes from unswollen (undifferentiated) stolons to swollen (differentiated) stolons and new tubers. There is an

evidence that the tuberization response is controlled by genetic processes (Ehlenfeldt and Hanneman, 1984). However, genes involved directly in early tuberization is unknown. Among floral homeotic genes, several genes are also expressed in vegetative organs including non-MADS-box genes such as *NFL 1* and *2* of tobacco (Kelly et al., 1995), *FLO* of *Antirrhinum* (Carpenter and Coen, 1990), and *LFY* of *Arabidopsis* (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992), and MADS-box genes such as *AGL3* of *Arabidopsis* (Ma et al., 1991), *TM3* of tomato (Pnueli et al., 1991), and *TobMADS1* of tobacco (Mandel. et al, 1994). The function of these homeotic genes is unknown, but it can be proposed that some homeotic genes are involved in regulating gene expression that leads to vegetative organ development during plant growth. Therefore, it is very likely that the morphological events of tuberization may be controlled by homeotic genes.

Objectives Of The Study

My research goals are to understand: (1) how plants accomplish differentiation and development, and (2) how genes are organized and involved in plant developmental processes. The main approach to study development is an analysis of molecular and genetic processes related to morphogenesis.

The main objective of my Ph.D. research project is to isolate and characterize genes involved in plant vegetative tissue development using potato tuberization as a model system. This has been accomplished by: (1) isolating and characterizing cDNAs from an early tuber cDNA library and (2) investigating the patterns of gene expression during the development of vegetative organs.

Dissertation Organization

This dissertation contains four journal papers prepared for publication. Chapter 1 is the General Introduction. Chapters 2 and 3 have been published in the journals, *Plant Physiology,*

and *Plant Cell Reports*, respectively. Chapter 4 is in press in the journal, *Gene*. Chapter 5 was prepared to be submitted in the journal, *Plant Molecular Biology*. Authorizations for copyright transfer have been permitted by each of the publishing companies. Chapter 6, General Summary, is the last chapter of the dissertation. The references cited in chapters, 1 and 6, are given in the Literature Cited section following Chapter 6. Chapters, 2, 3, 4, and 5, contain their own citations. I performed all of the research under the supervision of Dr. David J. Hannapel in the Department of Horticulture, Iowa State University, from the Fall of 1991 to the Spring of 1995 as a member of the Interdepartmental Major Plant Physiology.

CHAPTER 2. NUCLEOTIDE SEQUENCE OF A cDNA FOR THE POTATO (*Solanum tuberosum* L.) CHLOROPLAST RIBOSOMAL PROTEIN S16[1]

A paper published in *Plant Physiology* 107: 293-294 (1995)

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Plant chloroplast genes encode rRNAs, tRNAs, and proteins for their own transcription and translation systems. Plant chloroplast ribosomes, which are a part of the translational apparatus, are prokaryotic-like, 70S in size, and comprised of 16S, 23S, 5S, and 4.5S rRNAs and about 58 - 62 different ribosomal protein species (Subramanian 1985). Nineteen to twenty different ribosomal protein genes which code 11 or 12 proteins of the small ribosomal subunit and 8 proteins of the large subunit are located in the chloroplast genome (Shinozaki et al. 1986 b). Here we report the full-length sequence of a cDNA encoding the potato (*Solanum tuberosum* L.) chloroplast ribosomal protein S16 (cp *rps16*). The deduced amino acid sequence of cp *rps16* gene is over 80% conserved among higher plants, but is less than 30% conserved between plants and *E.coli* (Kanakari et al. 1992). Stern et al. (1988) reported that *E. coli* ribosomal protein S16 was associated with other ribosomal proteins (S4, S8, and S20) to facilitate the binding and assembly of the 16S rRNA into the 30S subunit.

Using conventional differential screening techniques, we have obtained several early tuber cDNA clones from a 4-day axillary bud-tuber λZAP library. One of these clones, designated p24-2, contained an approximately 1.6 kb insert and was sequenced in its entirety.

As shown in Figure 1, The 1561 bp sequence showed high homology with known chloroplast *rps16* genes, which code for chloroplast ribosomal protein S16 of tobacco, mustard, rice and maize (Shinozaki et al. 1986 a and b; Neuhaus et al. 1989; Hiratsuka et al. 1989; Kanakari et al. 1992), respectively. Based on the DNA sequence similarity and its amino acid sequence analysis, we concluded that the cDNA p24-2 was the potato chloroplast *rps16* gene. This potato cp *rps16* gene is located between *trnK* gene for $tRNA^{lys}$ (UUU) and trnQ gene for $tRNA^{gln}$ (UUG) on the same strand in the chloroplast genome. The 5' untranslated sequence contains an AT-rich region of 239 nucleotides and a putative ribosome binding sequence element 5'-AGGA-3' that is located five nucleotides upstream from the initiation codon, ATG. Two reading frames are interrupted by an intron of 855 nucleotides. Exon 1 is 40 bp long and extends from the nucleotide position 294 to position 333 and encodes for 13 amino acids. Exon 2 consists of 227 bp, encodes for 75 amino acids, and is located from the nucleotide position 1189 to a termination codon TAA at position 1415. The intron interrupts the CGA arginine codon between C and G. No putative open reading frame is contained in the intron. This structure is similar to that found in tobacco (Shinozaki et al. 1986 a) and in mustard chloroplast *rps16* (Neuhaus et al. 1989). The 5' end, 5'-GTGCGACTTG-3', and the 3' end, 5'-TCTATCCCAAT-3', of the intron boundary sequences of the potato cp *rps16* are highly conserved among mustard, maize, and tobacco. Both the 5' and 3' boundary sequences of the potato cp *rps16* intron showed typical characteristics of related intron types of group II, including introns of tobacco cp *rps12*, *rpl2*, and *rpl16* genes (Ohto et al. 1988). The 3' end of the untranslated sequence consists of 146 bp and shows typical plastid gene construction in that it contains no polyadenylation signal, but instead has several inverted repeat sequences, which could form a hairpin structure in their DNA and a stem-loop structure in their transcripts. The inverted repeats include one 5' to 3' direction element 5'-GGAAATACAAAAAA-3' with a completely inverted complement of this sequence, and three different sets of inverted direction elements. The putative stem-loop structure created by 3' inverted repeats is typical of plastid

mRNA. These inverted repeat sequences resemble prokaryotic terminators and may function as termination signals during chloroplast transcription (Sijben-Müller et al. 1986). In contrast, it was suggested that the 3' inverted repeat elements of other chloroplast genes were ineffective as transcription terminators, but instead functioned to increase the stability of chloroplast transcripts (Stern and Gruissem 1987). The potato cp *rps16* gene has properties of both prokaryotic [Shine-Dalgarno element, no poly(A^+) sequence] and eukaryotic (intron sequence) gene structure.

Footnotes

[1] This work was supported by the Iowa State University Biotechnology Council. Journal Paper No. J-15960 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project No. 3056.

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Abbreviations: cp, chloroplast; *rps*, ribosomal protein small subunit.

The GenBank accession number for the sequence reported in this article is U11638.

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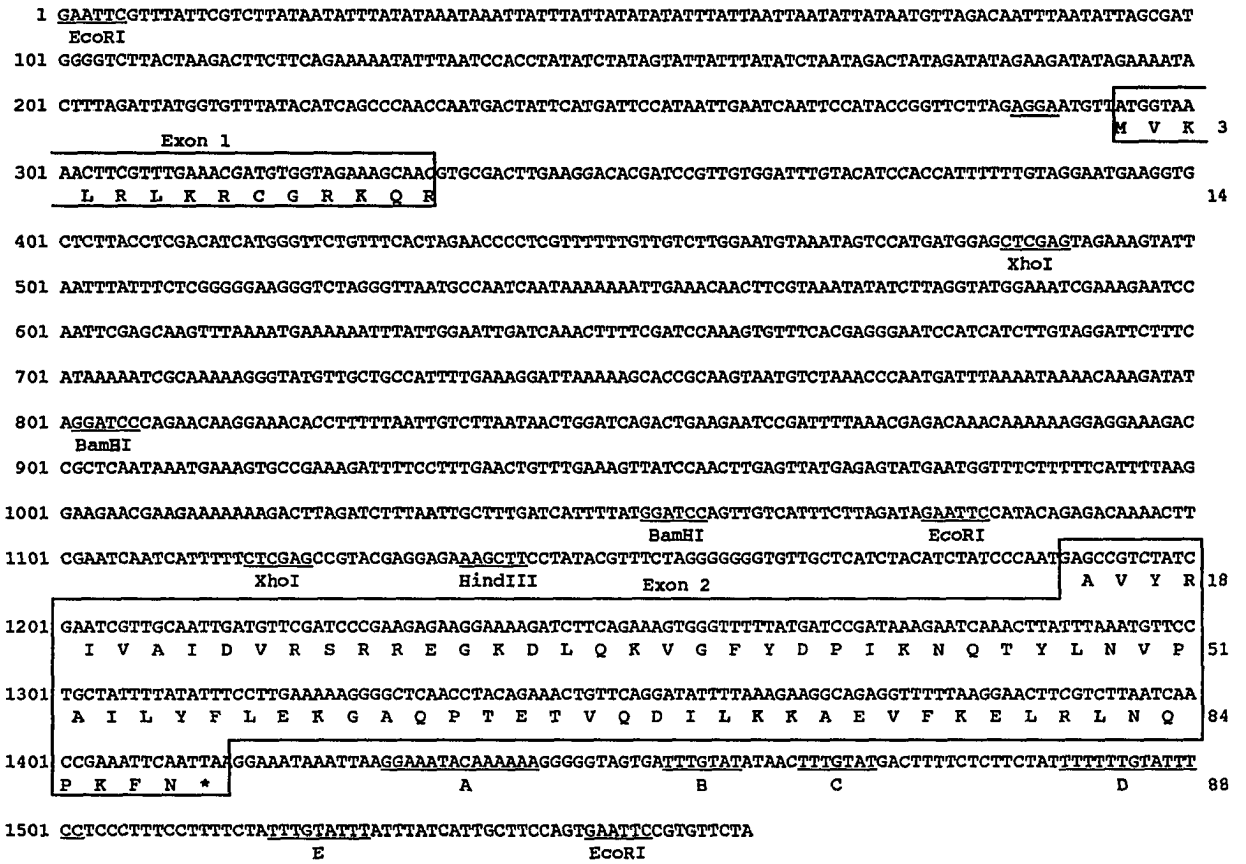

Fig. 1. DNA sequence of the potato chloroplast *rps16* cDNA, p24-2. The two exons are *boxed* with the deduced amino acid sequence shown. The putative Shine-Dalgarno sequence is *underlined* 5 bases upstream of the initiation codon. The *underlined* 3' sequences designated *A, B, C, D,* and *E* indicate inverted repeats in the 3' untranslated region.

Table I. *Characteristics of a cDNA for potato chloroplast ribosomal protein S16*

Organism:

Potato (*Solanum tuberosum* L. cv Superior).

Genome Location:

Between the *trnK* gene for $tRNA^{Lys}$(UUU) and *trnQ* gene for $tRNA^{Gln}$(UUG) on the same strand in the chloroplast genome.

Gene Product:

Chloroplast ribosomal protein S16 (cp RPS16).

Source:

cDNA library in lZAPII constructed from poly(A)$^+$RNA isolated from 4-day axillary bud-tuber. It is proposed that the 3' end containing several inverted repeat sequences allowed cDNA synthesis.

Techniques:

Differential screening techniques, restriction fragment subcloning into pGEM11Z, dideoxy sequencing of both strands of overlapped fragments.

Methods of Identification:

Comparison of the published cp rps16 gene and its amino acid deduced sequences of tobacco (Shinozaki et al. 1986 a), mustard (Neuhaus et al. 1989), and maize (Kanakari et al. 1992).

Features of cDNA structure:

Total length of 1561 bp, representing a full-length unprocessed transcript containing a group II intron of 855 bp, exon-1 of 40 bp, exon-2 of 227 bp, AT-rich 239 bp of the 5' untranslated sequence containing a ribosomal binding sequence element 5'-AGGA-3', a 3' untranslated region of 146 bp containing five inverted repeat sequences. No poly(A)$^+$ tail observed.

Features and Function of Deduced Amino Acid Sequence:

Two exons encode an 88-amino acid sequence of RPS16. The 13 amino acids encoded by exon-1 are highly conserved among higher plants. Homology of the cp RPS16 to other plants showed 95% with tobacco, 90% with maize, 41% with *Cynadium caldurium*, and 33% with *E. coli.* Function of RPS16 is associated with ribosomal proteins S4,

S8, and S20 to facilitate the binding and assembly of the 16S rRNA into the 30S subunit (Stern et al. 1988).

Subcellular Location:

Chloroplast.

Expression Characteristics:

Two transcripts, about 1.5 kb immature and 0.7 kb mature RNAs have been identified in chloroplast. The expression is developmentally regulated, with transcript levels decreasing during tuberization and increasing during shoot and leaf growth. Accumulation of S16 transcripts is constitutive in proplastids, reduced in amyloplasts, and enhanced by light in chloroplasts.

CHAPTER 3. CHARACTERIZATION OF A cDNA FOR CHLOROPLAST RIBOSOMAL PROTEIN S16 OF POTATO (*Solanum tuberosum* L.)

A paper published in *Plant Cell Reports* 14: 359-365 (1995)

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Summary

A cDNA encoding the potato (*Solanum tuberosum* L.) chloroplast ribosomal protein S16 (cp *rps*16) was isolated and characterized. The deduced amino acid sequence of the chloroplast *rps16* coding sequence from potato shows 33% homology to the *E.coli* ribosomal protein S16 and 95% homology to the tobacco chloroplast ribosomal protein S16. The 13 amino acids encoded by exon-1 are highly conserved (100% identity) among higher plants. Two sizes of *rps16* transcripts have been identified from northern blot analysis. The 1.5 kb transcript contains the 855 bp intron and represents an unprocessed RNA molecule, whereas the 0.7 kb transcript represents the mature form. The expression of *rps16* is developmentally regulated, with transcript levels decreasing during tuber development and increasing during shoot development. The potato cp *rps16* gene appears to be constitutively expressed in unswollen stolons and axillary buds, and its expression is enhanced by light.

Key words: Potato (*Solanum tuberosum* L.) - Chloroplast ribosomal protein S16 - *rps16* gene - Intron - Tuber development

Abbreviations. cp: chloroplast; *rps*: ribosomal protein small subunit

Introduction

Plant chloroplast genes encode rRNAs, tRNAs, and proteins for their own transcription and translation systems. Plant chloroplast ribosomes, which are a part of the translational apparatus, are prokaryotic-like, 70S in size, and comprised of 16S, 23S, 5S, and 4.5S rRNAs and about 58 - 62 different ribosomal protein species (Capel and Bourque 1982; Subramanian 1985). Nineteen to twenty different ribosomal protein genes which code 11 or 12 proteins of the small ribosomal subunit and 8 proteins of the large subunit are located in the chloroplast genome (Shinozaki et al. 1986 b). One of these proteins, ribosomal protein S16 (*rps16* gene) has been studied in *Nicotina tabaccum* (Shinozaki et al. 1986 a and b), *Sinapis alba* (Neuhaus et al. 1989), *Zea mays* (Kanakari et al. 1992), *Cyanidium caldarium* (Maid et al. 1992), and *Hordeum vulgare* (Sexton et al. 1993). We have recently reported the full-length sequence of a cDNA encoding the potato (*Solanum tuberosum* L.) chloroplast ribosomal protein S16 (cp *rps16*) (Kang and Hannapel, 1994).

The chloroplast *rps16* gene of higher plants is located between the *trnK* gene for $tRNA^{lys}(UUU)$ and the *trnQ* gene for $tRNA^{gln}(UUG)$ on the chloroplast genome (Shinozaki et al. 1986 b). The structure of cp *rps16* gene has characteristics of both prokaryotic and eukaryotic genes. The cp *rps16* gene has the inverted repeat sequences immediately proximal to the transcriptional termination region and its promoter has the consensus motifs similar to those of the prokaryotic "-35 region (TTGaca)" and '-10 region (TAtaaT)" (Shinozaki et al. 1986 a; Kanakari et al. 1992). Many chloroplast ribosomal genes of the tobacco chloroplast

are transcribed as a cluster in a prokaryotic manner similar to that of the homologous genes in *E. coli,* e.g., *S10* and *spc* operons (Tanaka et al. 1986), whereas cp *rps16* gene is transcribed as single cistronic transcription unit (Shinozaki et al. 1986 a; Neuhaus et al. 1989). In addition to its many prokaryotic features, the coding sequence of the cp *rps16* gene is interrupted by an intron (Shinozaki et al. 1986 a; Neuhaus et al. 1989; Kanakari et al. 1992; Sexton et al. 1993), a structural feature of eukaryotic genes. The deduced amino acid sequence of cp *rps16* gene is over 80% conserved among higher plants, but is less than 30% conserved between plants and *E.coli* (Kanakari et al. 1992). The function of chloroplast ribosomal protein S16 is not known, but Stern et al. (1988) reported that *E. coli* ribosomal protein S16 was associated with other ribosomal proteins (S4, S8, and S20) to facilitate the binding and assembly of the 16S rRNA into the 30S subunit. It has been suggested that the pattern of expression is developmentally regulated and light-independent. Neuhaus et al. (1989) reported that mustard cp *rps16* gene was constitutively expressed at the RNA level under either dark or light during seedling development. In contrast, barley cp *rps16* gene expression is abundant early in chloroplast development and then decreases in mature chloroplast (Baumgartner et al. 1993).

In this study, we report the characterization of the chloroplast ribosomal protein S16 of potato (*Solanum tuberosum* L.). Using northern blot analysis, we have examined the pattern of cp *rps16* gene expression during development and in response to light and high sucrose levels.

Materials and methods

Plant material and axillary bud tuber development. Potato (*Solanum tuberosum* L. cv. Superior) plants were grown in a greenhouse under an 18 h photoperiod. When plants reached 30 cm in height, they were grown under short-day conditions (8 h photoperiod) for a period of 3 weeks. Petiole-leaf cuttings having a single axillary bud were excised and cultured in a

perlite : vermiculite medium (3:1 v/v), with the bud completely covered by the medium. Cuttings were grown in a growth chamber under 8 h of cool white Sylvania fluorescent light (200 $\mu mol \cdot s^{-1} \cdot m^{-2}$)/18° C and 16 h dark/15° C to induce tuber formation in axillary buds (Hannapel 1991). Axillary buds were collected directly from the whole short-day treated plants at this time and were designated as 0 day buds. Induced axillary buds were harvested by excising the bud from the leaf-petiole cuttings at 2, 4, 8, and 16 days after the beginning of culture. Mature tubers, stems, leaves and roots were collected from plants grown in the greenhouse. Unswollen stolons and new tubers, characterized by a 2-3 mm diameter swelling at the tip of stolon, were harvested from whole plants 4 to 5 weeks after planting. All samples were frozen in liquid nitrogen immediately after harvest and stored at -80°C.

Tuber sprout culture. Sprouts were collected from seed tubers stored in the dark at 24° C. Sprout sections (about 1cm in length) with one axillary bud attached were cultured on solidified (0.7% agar) MS medium (pH 5.8) with either 1.0 or 8% (w/v) sucrose without any growth regulator. Both high and low sucrose-treated sprout cuttings were grown in growth chambers under both light (8h 200 $\mu mol \cdot s^{-1} \cdot m^{-2}$ of light/20° C; 16h dark/15° C) and dark (20° C for 8 h ; 15° C for 16h) conditions for 3 weeks. At the time of harvest, samples were frozen in liquid nitrogen and stored at -80° C.

Northern blot analysis. Total RNA was extracted from frozen samples using the phenol/chloroform extraction method (Hansen and Hannapel, 1992). Ten micrograms of total RNA was separated electrophoretically on 1.4% denaturing agarose gels using 5mM methyl mercury hydroxide (Alfa Products, Danvers, MA, USA) in 1x Tris-borate buffer (Sambrook et al. 1989). Each gel was stained with ethidium bromide and visualized under UV light to confirm that equal amounts of RNA were loaded in all lanes, and then was blotted onto nylon membrane (Schleicher and Schuell, Keene, NH, USA). Using differential screening

techniques, several cDNA clones (pBluescript SK-) were isolated from a 4-day axillary bud-tuber λZAP library (Stratagene Inc., LaJolla, CA, USA). One of these clones, designated p24-2 (Kang and Hannapel, 1994), was selected for further study. Inserts were gel-purified, labeled with [α-^{32}P] dATP by nick translation (Promega Inc., Madison, WI, USA), and used in northern blot analysis to study transcript types and the expression of *rps16* gene during development. The following probes were used: the 484 bp EcoRI-XhoI fragment for use as an exon 1-specific probe, the 593 bp XhoI-EcoRI fragment for use as an intron-specific probe, the 411 bp HindIII-EcoRI fragment for use as an exon 2-specific probe (Fig. 2A), and the entire 1561 bp insert of p24-2 for the developmental studies (Figs. 3 and 4). A patatin cDNA, pGM01 (Mignery et al. 1984), was used as a developmental control. Procedures for hybridization, washing, and exposure to film were carried out as described by Suh et al. (1991).

Results

Deduced amino acid sequence analysis of potato chloroplast rps16

The 88 deduced amino acids of potato cp *rps16* gene were aligned with related sequences and showed 95%, 90%, and 82% homology to chloroplast ribosomal protein S16 of tobacco (Shinozaki et al. 1986 a), maize (Kanakari et al. 1992), and mustard (Neuhaus et al. 1989), respectively, and 41% and 33% homology to those of *Cyanidium caldarium* (Maid and Zetsche 1992) and *E.coli* (Byström et al. 1983), respectively (Fig. 1). The last two organisms have an uninterrupted *rps16* gene. The deduced amino acid sequence of exon 1 (the consensus 13 amino acids MVKLRLKRCGRKQ) showed 100% identity to that of cp *rps16* genes of tobacco (Shinozaki et al. 1986 a), mustard (Neuhaus et al. 1989), and maize (Kanakari et al. 1992). The N-terminal 13 amino acids show 77% and 61.5% identity to the ribosomal protein

S16 of *Cyanidium caldarium* (Maid and Zetsche 1992) and *E. coli* (Byström et al. 1983), respectively.

Potato plastid rps16 expression during development

To study characteristics of *rps16* transcript accumulation during plastid differentiation and plant development, northern blot analysis was performed using RNA from various organs of the potato plants as well as developing buds from a model system we use to control tuber growth (Hannapel 1991). Northern blot analysis was carried out on total RNA extracted from underground unswollen stolons, newly formed tubers (<0.5g), mature tubers (>50g), and leaves from whole plants. Exon 1-specific (484 bp EcoRI-XhoI fragment), exon 2-specific (411bp HindIII-EcoRI fragment), and intron-specific (593 bp XhoI-EcoRI fragment) probes were prepared from cDNA p24-2 (Fig. 2A). Patatin (pGM01), the major storage protein, was used as a control probe of tuberization. For all tissue types, both exon 1- and 2-specific probes hybridized to RNA bands of about 1.5 kb and 0.7 kb (Fig. 2B-I and III). In contrast, the intron-specific probe hybridized only to the 1.5 kb transcript (Fig. 2B-II). It is likely that the 1.5 kb molecule is the immature, unspliced transcript and that the 0.7 kb molecule is the mature, spliced transcript. For both the intron- and exons-specific probes, levels of *rps16* transcripts gradually decreased as the tubers enlarged, and were highest in green leaves (Fig. 2B). When the exon 2-specific probe was used, the 1.5 kb and 0.7 kb transcripts were detected in proportionately equal amounts in unswollen stolons, newly formed tubers and leaves (Fig. 2B-III). The 1.5kb transcripts were more abundant than the 0.7 kb transcripts in mature tubers (Fig. 2B-III), indicating that some post-transcriptional regulation might be involved. As expected, patatin transcript levels dramatically increased from unswollen stolons to newly formed tubers and mature tubers and only trace amounts of patatin transcripts were detected in leaf samples (Fig. 2B-patatin).

To further characterize *rps16* expression during development, we made use of an axillary bud tuber system from petiole-leaf single-node cuttings (Ewing 1985; Hannapel 1991) as a model for studying organ and plastid development. Axillary bud morphogenesis proceeded through the following sequence from dormant axillary buds to micro-tubers: day 0 - green-colored dormant axillary buds; day 2 - green-colored buds (axillary buds beginning growth); day 4 - green-colored buds (tuber differentiation beginning); day 6 - greenish-white and swollen buds (initiation of tuber morphogensis); day 8 - whitish, swollen bud tubers (5-7 mm diameter in size); and day 16 - white micro-tuber (12-15 mm diameter in size). This model system mimics the morphological and biochemical processes of whole plant tuberization (Hannapel 1991). A patatin cDNA probe was used in the northern blot hybridization as a marker for tuberization. Patatin transcripts were not detected in total RNA extracted from 0-day axillary bud cuttings, stem, leaf and root tissues (Fig. 3B). The level of patatin expression increased from day 2 through the mature tuber stage. Levels of *rps16* transcripts in axillary buds increased from day 0 to day 2, then gradually decreased through day 16 (Fig. 3A). In mature tubers, only trace amounts of unspliced transcript (1.5 kb) were detected and no mature transcript (0.7 kb) was detected (Fig. 3A). Both transcript types were detected at low levels in roots, whereas intermediate levels of both transcripts were present in stem tissue, and high levels detected in leaf samples (Fig. 3A).

The effect of light and sucrose on rps16 expression

We observed that samples obtained from underground structures, i.e., roots, stolons, and tubers, had lower levels of cp *rps16* transcripts, but leaves and day 2 axillary buds had higher levels (Fig. 2 and 3), indicating that light might induce *rps16*. Little difference was observed in the levels of cp *rps16* transcripts of mustard seedlings (Neuhaus et al. 1989) and tobacco leaves (Shinozaki et al. 1986 a) grown under light or dark conditions. Because several

potato tuber genes are induced by sucrose (Johnson and Ryan 1990; Suh et al. 1991), we also examined the effects of high sucrose on *rps16* expression *in vitro*. Northern blot analysis was used to characterize *rps16* gene expression under light and dark conditions. Total RNA samples were harvested from shoot or stolon tissue grown from potato tuber sprout segments cultured on high (8.0%) or low (1.0%) sucrose in dark or light conditions. Sprouts grown under dark conditions from seed tubers were white and elongated (Fig. 4, I-A). Axillary sprout buds differentiated after 3 weeks of culture as follows; green-colored shoots in 1% sucrose media under 8 h light (Fig. 4, I-B); elongated stolons (no chlorophyll visible) in 1% sucrose in the dark (Fig. 4, I-C); both green tubers and shoots in 8% sucrose media under 8 h light (Fig. 4, I-D); and small tubers (no chlorophyll visible) in 8% sucrose in the dark (Fig. 4, I-E). The most striking results of the northern blot analysis were the increase of *rps16* transcript levels in total RNA from the light grown tissues (compare lanes B and C, and lanes D and E in panel II-*rps16* of Fig. 4). High levels of sucrose did not increase *rps16* RNA levels (compare lanes C and E of Fig. 4, panel II-*rps16*), unless the tissue was exposed to light (Fig. 4, II-D). Light conditions were sufficient to increase cp *rps16* transcript accumulation even on the media with only 1.0% sucrose (Fig. 4, II-B). Both *rps16* transcript types accumulated to the same relative amounts in each of the tissue samples. Low levels of patatin mRNA were detected in tuber sprouts with the highest levels detected in the tissue samples cultured on high sucrose media (Fig. 4, panel II-Patatin, lanes A, D, and E, respectively). Previous work has clearly shown that patatin genes are sucrose-inducible (Wenzler et al. 1989).

Discussion

Northern blot analysis consistently showed the presence of two discrete transcripts for potato cp *rps16*, one of about 1561 nucleotides in length and another of approximately 700 nucleotides. Using two exon-specific probes and one intron-specific probe, we demonstrated

that the larger transcript represented an unspliced form containing the intron and the smaller transcript represented a mature, spliced form. In the tissues we examined, the two transcript types accumulated proportionately. For example, the highest levels of both transcripts were detected in leaf tissue. The lowest level of high molecular weight transcript accumulation occurred in roots and mature tubers. In these same tissues, the mature transcript form was barely detectable. Shinozaki et al. (1986 a) have suggested that splicing of the unprocessed immature transcript may function as a rate-limiting step in *rps16* gene expression. Like ribosomal protein transcripts of *E.coli* (Nomura et al. 1984) and yeast (Dabeva et al. 1986; Presutti et al. 1991), the splicing of potato cp *rps16* transcripts may be autogenously regulated to control gene expression. In this model, when the synthesis of the ribosomal protein is substantially increased, the excess ribosomal protein competitively binds to the immature *rps16* transcript types. Binding to the immature transcripts inhibits splicing and reduces the level of the translatable, mature transcript.

Proplastids, which are small, undifferentiated plastids, are present in all meristematic plant cells. These proplastids are capable of differentiation which varies greatly depending on cell type and organ function. Plastids in mature tuber and root are generally amyloplasts or chromoplasts, reflecting the storage function of these organs (Thomson and Whatly 1980; Marinos 1967), whereas the plastids of green leaf, shoot, and stem epidermis are photosynthetic chloroplasts (Mullet 1988). Our results indicate a correlation between plastid differentiation and *rps16* gene expression. It could be that *rps16* expression is induced by light because chloroplast differentiation from proplastids is induced by light. However, Neuhaus et al. (1989) reported that levels of mustard cp *rps16* transcripts increased initially and then remained constant in mustard seedlings grown under either light or dark conditions. Baumgartner et al. (1993) observed that *rps16* transcripts of barley chloroplast were more abundant in leaf sections of dark-grown plants compared with illuminated plants, and proposed that *rps16* transcription reached maximal abundance early in chloroplast development, before

accumulation of light-induced gene transcriptions (*rbcL, aptB, psaA*, and *petB* genes). Our results showed that there is constitutive expression of *rps16* in non-green organs like stolons and tuber sprouts. The induction experiments, however, with tuber sprout segments showed that *rps16* is enhanced by light but not affected by high sucrose levels. The *rps16* gene appears to play a part in house keeping functions of undeveloped plastids, but is most active during chloroplast development in response to light. The development of photosynthetic plastids from proplastids in the meristems of shoot apices and the leaf primordia involves the activation of numerous genes encoding photosynthetic polypeptides. In conjunction with this increase in metabolism would be the increase in ribosomal RNA synthesis as well as ribosomal-associated proteins like the cp ribosomal protein S16. The low expression of *rps16* in cells from developing tubers is a reflection of the role that plastid genes play in the development of amyloplasts. It has been suggested by Mullet (1988) that during proplastid differentiation in amyloplasts, RNA and protein synthesis are greatly reduced with concomitant reduction in the transcriptional and translational apparatus (including rRNA synthesis). Aguettaz et al. (1987) observed that plastid DNA copies per cell, 16S rRNA levels, and the cellular level of *rbcL* transcripts in spinach cell suspensions decreased during amyloplast development.

The results of this study indicate that the expression of the potato cp *rps16* gene is constitutive in proplastids, reduced in amyloplasts, and enhanced by light in chloroplasts and is dependent on the stage of cell and tissue differentiation.

Acknowledgments. We wish to thank Mary A. Tymeson for her excellent technical assistance.

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                          +
   Potato   1 MVKLRLKRCG RKQRAVYRIV AIDVRSRREG KDLQKVGFYD PIKNQ....T 46
  Tobacco   1 ********** ********** ********** ***R****** *****....* 46
  Mustard   1 ********** ********** ********** R**R****** **T**....* 46
    Maize   1 ********** ***Q*I**** ********** R**R****** *****....* 46
Cyanidium   1 *L*I****** **KQTSFK** VMNNLDK*D* QAIEEL**MN *RTKE....K 46
   E.coli   1 *VTI**A*H* A*K*PF*QV* VA*S*NA*N* RFIER***FN **ASEKEEG* 50

   Potato  47 YLNVPAILYF LEKGAQPTET VQDILKKAEV FKELRLNQPK FN 88
  Tobacco  47 ********** ********G* ********** *****P**S. .. 85
  Mustard  47 ***L****D* *K******R* *H**S***GI *T**N**KT* L* 88
    Maize  47 C********* ********R* *Y***R***F **DKERTLS. .. 85
Cyanidium  47 ***INK*NHY *RL**K**K* *F*LLN**KI I......... .. 77
   E.coli  51 R*DLDR*AHW VGQ**TISDR *AALI*EVNK AA........ .. 82
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Fig. 1. Comparison of the deduced amino acid sequence of potato chloroplast ribosomal protein S16 with those of the corresponding sequences of tobacco (Shinozaki et al. 1986 a), mustard (Neuhaus et al. 1989), maize (Kanakari et al. 1992), *Cyanidium* (Maid and Zetsche 1992), and *E.coli* (Byström et al. 1983), respectively. Identical amino acids are marked by *asterisks*, and the *Greek cross* (+) indicates the site of intron splicing.

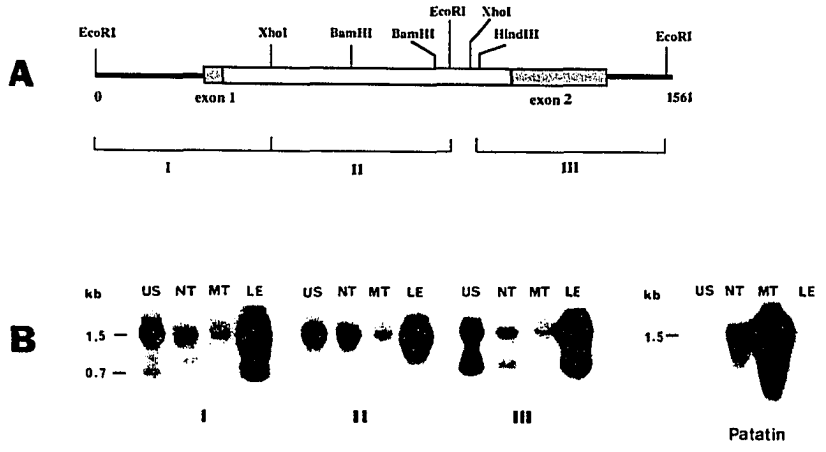

Fig. 2. Restriction map of potato cp *rps16* cDNA, p24-2 (A) and northern blot analysis (B). **A.** The two exons are represented by solid boxes and the intron is represented by the open box. Three regions of the sequence are designated: (I) the exon 1-specific EcoRI-XhoI fragment with a length of 484 bp's, (II) the intron-specific XhoI-EcoRI fragment of 593 bp's, and (III) the exon 2-specific HindIII-EcoRI fragment of 411 bp's. **B.** Northern blot analysis of *rps16* transcript levels during potato tuber development and in mature leaves. Total RNA was isolated from underground unswollen stolons (US), newly formed tubers (NT), mature tubers (MT), and mature leaves (LE). Panels I, II, and III show the results of northern blot analysis using different [^{32}P]-labeled probes, exon 1 probe (I), the intron probe (II), and exon 2 probe (III) of panel A. Labeled insert from patatin cDNA, pGM01, was used as a control. Ten μg of total RNA were loaded for each sample.

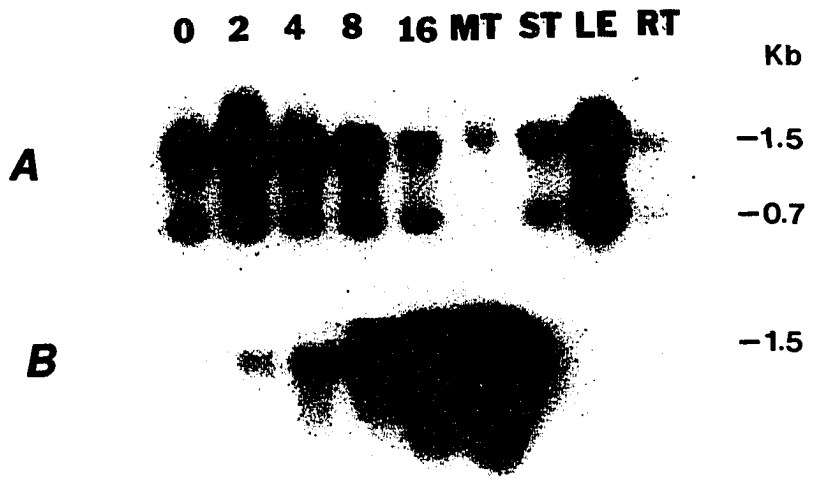

Fig. 3. Northern blot analysis of total RNA extracted from developing axillary bud tubers harvested after 2,4, 8, and 16 days of culture. Petiole-leaf cuttings with the axillary bud intact were taken from stock plants (potato cultivar Superior) grown in the greenhouse under a short-day photoperiod. Zero day buds (0) were excised directly from the stock plant. Lanes MT, ST, LE, and RT represent total RNA extracted from mature underground tuber, stem, leaf, and root tissue, respectively. The filters were hybridized with [^{32}P]-labeled inserts for either the *rps16* cDNA, p24-2 (A) or patatin, pGM01 (B). Ten μg of total RNA were loaded for each sample.

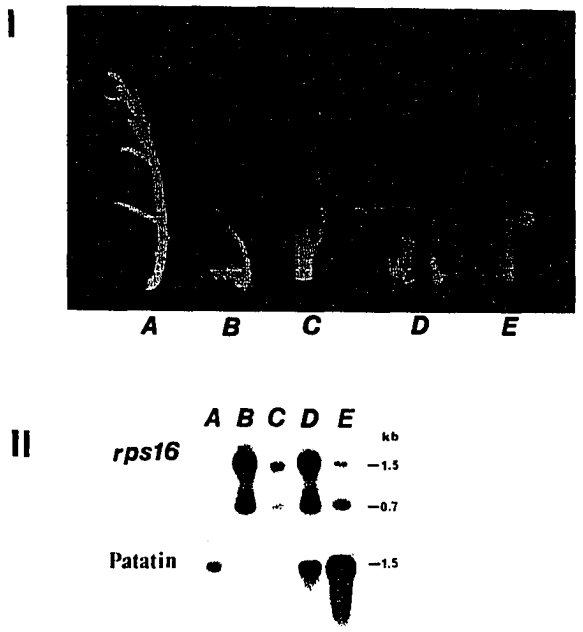

Fig. 4. Morphological response of axillary buds from tuber sprout explants cultured under various light/sucrose combinations (I) and northern blot analysis (II). **I.** Sprouts were collected from seed tubers stored in the dark for several weeks at 24° C. Sprout segments (1cm in length) with one axillary bud attached were excised and cultured on semisolid MS medium under the following conditions: 8h 200 mmol·s^{-1}·m^{-2} of light/20° C; 16h dark/15 ° C in 1% (B) or 8% (D) sucrose; and dark (20° C for 8 h ; 15° C for 16h) in 1% (C) and 8% (E) sucrose. Intact, dark-grown tuber sprouts that received no treatment (A) were included for comparison. **II.** Northern blot analysis of total RNA extracted from tuber sprout explants shown in panel I. The filters were hybridized with [^{32}P]-labeled cDNA inserts for *rps16*, p24-2 or patatin, pGM01. Ten μg of total RNA were loaded for each sample.

CHAPTER 4. SEQUENCES OF NOVEL POTATO (*Solanum tuberosum* L.) MADS-BOX cDNAS AND THEIR EXPRESSION IN VEGETATIVE ORGANS*

A paper accepted in *Gene* (May 30, 1995)

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(Homeotic gene; DNA-binding domain; plant transcription factor; plant vegetative organ development)

Summary

Two similar, apparently full-length potato MADS-box gene cDNAs from an early tuber cDNA library have been identified and sequenced. These two cDNAs, *POTM1*-1 and *POTM1*-2, encode 250 identical amino acids (aa) including 56 aa of the MADS-box domain and 53 aa of the K-box domain, indicating that they may function as transcription factors. The transcripts of these cDNAs accumulate abundantly in vegetative organs, suggesting that these novel MADS-box genes may be involved in vegetative organ development of potato.

Abbreviations: aa, amino acid(s); *AGL3*, *Arabidopsis AGAMOUS*-like gene 3; *AP1*, *Arabidopsis APETALA1* gene; bp, base pair(s); cDNA, DNA complementary to RNA; CDS, coding sequence(s); kb, kilobase(s); nt, nucleotides; ORF, open reading frame; PCR, polymerase chain reaction; *st-Def*, *Solanum tuberosum Deficiens* gene; *TM3*, Tomato gene 3;

TM4, Tomato gene 4; TobMADS1, tobacco MADS box gene; *POTM1*, potato MADS box gene 1; UTR, untranslated region(s).

Introduction

MADS-box genes are homeotic genes that have been isolated and characterized from several higher plants and that contain a conserved 56 aa DNA-binding domain referred to as the MADS-box and a conserved amphipathic alpha helix domain referred to as the K-box (keratin-like box) (Schwartz-Sommer et al., 1990; Ma et al., 1991). Analysis of the induced floral mutations of *Arabidopsis* (Coen and Meyerowitz, 1991) and *Antirrhium* (Schwarz-Sommer et al., 1990) has revealed that the putative transcription factors encoded by MADS-box genes play an important regulatory role in flower development. Among many floral MADS-box genes, however, only *AGL3* in *Arabidopsis* (Ma et al., 1991), *TM3* in tomato (Pnueli et al., 1991), and *TobMADS1* in tobacco (Mandel et al, 1994) are expressed in both floral and vegetative organs. Here we report the isolation and characterization of novel potato MADS-box gene cDNAs that are expressed abundantly in vegetative organs.

Experimental And Discussion

A cDNA library was constructed in λ ZAPII vector (Stratagene, La Jolla, USA) from mRNA extracted from 4-day-old axillary bud tubers of petiole-leaf cuttings of *Solanum tuberosum* cv 'Superior' grown under an 8 hr photoperiod (Hannapel, 1991). Two PCR oligonucleotide primers were designed based on the conserved MADS-box regions from floral MADS-box genes, and used to amplify a 130 bp fragment from 4-day-old axillary bud tuber cDNA as a template. This fragment was ^{32}P-labeled and used as a probe to screen the 4-day-old axillary bud tuber cDNA library. From approximately 40 cDNA clones isolated from this

library, two different groups of clones were identified; the pSK102 group that contained an internal *PstI* restriction enzyme site, and the pSK043 group that did not contain the *PstI* site. One clone from both groups was sequenced and characterized for further study. DNA sequence analysis revealed that both cDNAs were full-length and 99 % identical. The largest ORF of each cDNA encodes the same deduced 250 aa (approximately 29 KD) and contains 56 aa of the MADS-box domain in the N-terminal region and 38 aa of the K-box domain in the middle region (Fig.1). These two cDNAs likely represent different alleles of a potato MADS-box (*POTM*) gene family designated *POTM1*-1 from the pSK102 group and *POTM1*-2 from the pSK043 group. *POTM1*-1 is 1,085 base pairs (bp) in length and includes a 57 bp 5'-UTR, a 750 bp CDS, and a 278 bp 3'-UTR. A typical eukaryotic poly $(A)^+$ signal 5'-AATAAA is located in the 3'-UTR and is followed by a 17 bp poly $(A)^+$ tail. Microsatellite-like AT and CT dinucleotide repeats are located from nucleotide position 858 to 919 in the 3'-UTR (Fig.1). These short repeat sequences have not been found in any other homeotic genes, including those in the MADS-box gene families. These dinucleotide repeat sequences are usually contained within the 5'-upstream region or within introns of genomic sequences (Kondo et al., 1989), and may influence gene transcription (Naylor and Clark, 1990). However, the function of AT repeat sequences in 3'-UTR has not yet been characterized. *POTM1*-2 is similar to *POTM1*-1, but has a 14 bp insertion in the AT repeat sequences, no *PstI* site, and 6 mismatches in the entire sequence compared to *POTM1*-1 (Fig.1). Sequence comparison with other MADS-box genes showed 84.9 % homology to *TM4* of tomato (Pnueli et al., 1991), 55.6 % homology to *TM3* of tomato (Pnueli et al., 1991), 49.2 % homology to *AP1* of *Arabidopsis* (Mandel et al., 1992), 48.4 % homology to *tobMADS1* of tobacco (Mandel et al., 1994), and 45.8 % homology to *st-DefpD4* of potato (Gracia-Maroto et al., 1993).

Northern blot analyses using poly $(A)^+$ mRNA isolated from various potato tissues were performed and repeated several times under high stringency conditions. A PCR-

generated 170 bp fragment from the 3'-UTR was cloned into the pCR II vector (Invitrogen, San Diego, USA) and used as a template to generate a gene-specific RNA probe to eliminate possible cross-hybridization of probes to related MADS-box transcripts. Transcripts (approximately, 1,100 nts in length) of *POTM1* were abundant in axillary buds, leaf, 2-day and 4-day tuber-induced axillary buds (Hannapel, 1991), and root tissues, respectively (Fig. 2). Relatively lower levels of mRNA were detected in early flower, mature tuber, and stem tissues (Fig. 2). *POTM1* shows higher homology to floral-specific MADS-box genes such as *TM4* (84.9 % homology) than to the floral-vegetative types such as *TM3* (55.6 % homology) and *tobMADS1* (48.4 % homology) among *Solanaceous* species. However, transcripts were most abundant in vegetative organs, indicating that the expression pattern of *POTM1* is unique among MADS-box genes characterized to date. In addition, there is no significant homology of *POTM1* to other plant homeotic genes that are expressed in vegetative organs. In summary, we report that two novel MADS-box gene cDNAs, *POTM1*-1 and -2, are abundant in vegetative organs, suggesting these cDNAs might be involved in vegetative organ development.

Acknowledgements

We thank Mary Tymeson for technical support and Loren Stephens and Martin Spalding for reviewing the mauscript. This work was supported in part by the Iowa State University Biotechnology Council. Journal Paper No.16305 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project No. 3056.

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*On request, the authors will supply detailed experimental evidence for the conclusions reached in this Short Communication.

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Fig. 1. Nucleotide sequence alignment of potato MADS-box cDNAs, *POTM1*-1 (GenBank accession No. U23757) and *POTM1*-2 (GenBank accession No. U23758), and the deduced amino acid sequence. A blank space aligned in *POTM1*-2 indicates an identical base to that in *POTM1*-1; only divergent bases are indicated in alignment; dots indicate a deletion; the dinucleotide repeat is italicized in the 3'-UTR; and the *Pst*I site (5'-CTGCAG) is underlined at nucleotide position 370 of *POTM1*-1. The MADS-box domain is double underlined and the K-box domain is single underlined. Poly (A)+ signal is dot-underlined in the 3'-UTR.

```
                                  1
POTM1-2  ......................ATT                                          38
POTM1-1  TATATATATTACAAAAAACATTATTCTTTTTACTATCATTAAAAATTATCCGTAAAAATG        60
                                                                    M         1
                                                                             98
GGAAGAGGAAGAGTCCAGTTGAAGCGAATAGAGAACAAAATTAACCGTCAAGTGACTTTC               120
G  R  G  R  V  Q  L  K  R  I  E  N  K  I  N  R  Q  V  T  F                 21
                                  MADS box                                  158
TCGAAACGTCGATCTGGTTTGCTGAAGAAAGCTCATGAGATCTCTGTGCTTTGTGATGCT              180
S  K  R  R  S  G  L  L  K  K  A  H  E  I  S  V  L  C  D  A                 41
                                                                            218
GAGGTTGGTTTGATTGTTTTTTCCACTAAAGGAAAACTCTTTGAATATGCCAATGATTCA              240
E  V  G  L  I  V  F  S  T  K  G  K  L  F  E  Y  A  N  D  S                 61
                                                                            278
TGCATGGAGAGGTTACTTGAAAGATATGAAAGATACTCATTTGCTGAGAGGCAGCTTGTT              300
C  M  E  R  L  L  E  R  Y  E  R  Y  S  F  A  E  R  Q  L  V                 81
                                                                            338
CCTACTGATCATACATCCCCGGGAAGCTGGACTCTGGAACATGCAAAACTTAAGGCCAGA              360
P  T  D  H  T  S  P  G  S  W  T  L  E  H  A  K  L  K  A  R                 101
          T                                                                 398
CTTGAGGTTCTGCAGAGGAACCAAAAGCATTATGTGGGAGAAGATTTGGAGTCGTTAAAT              420
L  E  V  L  Q  R  N  Q  K  H  Y  V  G  E  D  L  E  S  L  N                 121
                                                                            458
ATGAAAGAACTTCAGAATCTTGAACACCAGCTTGATTCTGCTCTTAAACACATTCGATCA              480
M  K  E  L  Q  N  L  E  H  Q  L  D  S  A  L  K  H  I  R  S                 141
                                  K-box                                     518
AGAAAGAACCAATTGATGCATGAATCCATTTCTGTGCTTCAAAAACAGGACAGAGCATTG              540
R  K  N  Q  L  M  H  E  S  I  S  V  L  Q  K  Q  D  R  A  L                 161
                                                                            578
CAGGAGCAAAATAACCAGCTTTCCAAGAAGGTGAAGGAGAGGGAGAAAGAGGTGGCACAG              600
Q  E  Q  N  N  Q  L  S  K  K  V  K  E  R  E  K  E  V  A  Q                 181
                                                                            638
CAAAATCAGTGGGATCAGCAGAACCATGAAATCAACTCATCTACATTTGTTTTGCCACAA              660
Q  N  Q  W  D  Q  Q  N  H  E  I  N  S  S  T  F  V  L  P  Q                 201
                                                                            698
CAATTGGACTCTCCTCACCTTGGGGAAGCATACCAGAATACTAATGTAGTAGATAATGGA              720
Q  L  D  S  P  H  L  G  E  A  Y  Q  N  T  N  V  V  D  N  G                 221
                                                                            758
GAAGTGGAAGGAGGTAATTCTTCACAGCAGCAAGGTGCAGCTAATAATACTGTGATGCCA              780
E  V  E  G  G  N  S  S  Q  Q  Q  G  A  A  N  N  T  V  M  P                 241
                                                                            818
CAATGGATGCTTCGCCATCTTAATGGTTAAAAGTACCACTAATACTTGATGGTCACTATC              840
Q  W  M  L  R  H  L  N  G  *                                               250
                                                                   TATCTA   878
AATATAATTACCTAGGTATATATATATATATATATATATATATATATATATATATA.....              894

TATATATC                        A A A                                      938
........TATATATCTATCTCTCTCTCTCTCTATATTGATGCCACAATATATTGTCTAA               946
                                                                            998
ACGTACCTTCGAAGACATGCCACAATATAGATGGATTATTGTAAAAATATTATAGTATAT              1006
                                                                           1058
GTTAAAATGAACTTAAATGTATGATTTTCGCTACAACAAATAAAGGATTTTGCAGTTGTT              1066

  ATAAAAA*                                                                 1067
AT..AAAAAAAAAAAAAAAA*                                                      1085
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Fig. 2. Northern blot analysis of poly (A)+ RNA extracted from developing axillary bud tubers on petiole-leaf cuttings harvested after 2 and 4 days and other organs. Zero-day buds (axillary buds) were excised directly from the stock plants grown under a short-day photoperiod. Lanes MT, ST, LE, RT, and EF represent respectively, mature underground tuber, stem, leaf, root, and early flower (at floral stages from 1 to 12; Smyth et al., 1990) of potato cv. Superior. Filters were hybridized with [^{32}P-α-UTP]-labeled *POTM1*-specific RNA probe (170 bp 3'-UTR region). Four μg of poly (A^+) mRNA were loaded in each lane. An actin probe was used as a control to confirm uniform loading of mRNA in each lane (data not shown).

CHAPTER 5. THE MADS-BOX GENE *POTM1*-1 OF POTATO (*Solanum tuberosum* L.) IS EXPRESSED IN BOTH VEGETATIVE AND FLORAL ORGANS

A paper prepared to be submitted to *Plant Molecular Biology*, 1995

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Key words: homeotic gene, plant transcription factor, tuberization, vegetative organ development

Abstract

A potato MADS-box gene cDNA (*POTM1*-1) from an early tuber cDNA library has been isolated and characterized. The deduced amino acid sequence of *POTM1*-1 cDNA encodes 250 amino acids of a putative transcription factor containing a MADS-box domain and a K-box domain. These conserved domains share high homologies to those of flower-specific homeotic proteins, TM4 of tomato and AP1 of *Arabidopsis* , indicating that *POTM1*-1 gene is a homolog of the *AP1* gene family. The putative secondary structure in the K-box domain contains an amphipathic helix structure that is similar to those in other plant MADS-box proteins. A potential phosphorylation and four glycosylation sites are located in POTM1-1 protein, suggesting that posttranslational modification may be involved in the processing of POTM1-1. The levels of *POTM1*-1 transcripts were high in leaves, roots, aerial shoot tips, underground stolon tips, newly formed tubers, and late flowers, but relatively low in stems, early flowers,

and mature tubers. During axillary bud development in a model petiole-leaf cutting system, the levels of *POTM1*-1 transcripts were abundant in actively growing shoots and during the early stages of microtuber development. It is possible that POTM1-1 functions as a transcription factor that regulates plant developmental processes in a number of tissue types.

Introduction

Among floral homeotic genes, MADS-box genes [1] have been identified in numerous plant species [2, 3, 4, 5, 6, 7, 8]. MADS-box proteins are characterized by two conserved regions [1, 6]. One is the DNA-binding domain, designated the MADS-box [1], consisting of 56 amino acid residues of conserved motifs present in transcription factors of MCM1 in yeast [9], AG in *Arabidopsis* [4], DEF A in *Antirrhinum* [2], and SRF in human [10]. Using gel shift analysis and DNA foot printing, the DNA-binding domains of a heterodimer DEF A/GLO protein were shown to bind to a $CC(A/T)_6GG$ core element, designated the CArG box [11], in the 5' upstream region of their own genes [12, 13]. Immunolocalization studies have revealed that the flower MADS-box protein FBP1 of petunia [14] was detected in the nuclei of petal cells. This experiment supports the premise that MADS-box proteins function as DNA-binding proteins and transcription factors. The second conserved domain of MADS-box proteins is designated the K-box because of its structural similarity to the coiled-coil domain of keratin [6, 15]. It has been proposed that the K-box domain mediates protein-protein interactions of the MADS-box proteins to form homo- or heterodimers that facilitate DNA-binding with other regulatory factors [13].

Mutation analysis has shown that the MADS-box genes are involved in controlling flower development [2, 3, 4, 12, 16]. Floral organ homeotic genes can be categorized in three classes based on their mutant phenotypes [17]: 1) floral meristem identity genes involved in the formation of floral primodia such as *LFY*, *AP1* , and *CAL* in *Arabidopsis* [5, 18, 19] and *FLO*

in *Antirrhinum majus* [20]; 2) floral organ identity genes involved in the development of floral organs (sepals, petals, stamens, and carpels) such as *AP1*, *AP3* and *AG* of *Arabidopsis* [4, 5, 7] and *PLE, SQUA, DEF A, GLO* of *Antirrhinum majus* [2, 3, 12, 16]; and 3) cadastral genes such as *AG, SUP*, and *AP2* of *Arabidopsis* [4, 21, 22] and *PLE* of *Antirrhinum majus* [16]. Cadastral genes are involved in the regulation of organ identity genes. The consensus of this work supports the premise that floral homeotic proteins, including MADS-box proteins, act as enhancer or repressor factors that regulate gene expression controlling flower development.

Plant homeotic gene expression is not limited to floral organs, however. Many floral homeotic genes are also expressed in vegetative organs including non-MADS-box genes such as *NFL 1* and *2* of tobacco [23], *FLO* of *Antirrhinum* [20], and *LFY* of *Arabidopsis* [18, 24, 25], and MADS-box genes such as *AGL3* of *Arabidopsis* [6], *TM3* of tomato [26], and *TobMADS1* of tobacco [27]. It is likely that other homeotic genes are involved in regulating gene expression that leads to vegetative organ development during plant growth. Despite considerable efforts focusing on the molecular biology of the floral homeotic genes, however, very little is known about vegetative homeotic genes. To study plant vegetative development, potato (*Solanum tuberosum* L.) tuberization represents a useful model system. Tuberization involves a number of important biological processes such as carbon partitioning, starch metabolism, growth correlation, and signal transduction [28]. During the early stages of tuberization, differentiation of the stolon tip occurs by radial cell division and expansion and is accompanied by the accumulation of starch and a specific set of proteins [29, 30]. There is clear evidence that the tuberization response is controlled by genetic processes [31]. It is very likely that the homeotic genes are involving in regulating the complex morphological events of tuber initiation. Recently, we have identified a group of homeotic gene cDNAs represented by *POTM1* (potato MADS-box cDNA) from an early tuber cDNA library [32]. Three alleles of *Dificiens*-homologous potato floral-specific MADS-box genes (*St-Def pD13, pD10*, and *pD12*) were previously characterized [33]. Comparison of the nucleotide sequence of these *St-Def*

alleles to *POTM1* clearly shows that *POTM1* represents a unique family of potato homeotic genes that are differentially expressed in both vegetative and floral organs [32]. Like a typical MADS-box protein, POTM1 contains both MADS-box and K-box domains, indicating that the POTM1 protein may function as a transcription factor.

Materials and methods

Plant material and axillary bud tuber development

Potato (*Solanum tuberosum* L. cv. Superior) plants were grown in a greenhouse under an 18 hr photoperiod from seed tubers obtained from the Tatro Seed Co., Antigo, WI. When plants reached 30 cm in height, they were grown under either short-day conditions (8 hr photoperiod) or long-day conditions (16 hr photoperiod) for a period of 3 weeks. Petiole-leaf cuttings with a single axillary bud were excised and cultured in a petiole : vermiculite medium (3:1 v/v), with the bud completely covered by the medium. Cuttings for short-day were grown in a growth chamber under 8 hr of cool white Sylvania fluorescent light (200 $\mu mol \cdot s^{-1} \cdot m^{-2}$) at 18°C and 16 hr dark at 15°C to induce tuber formation in axillary buds. Details of this model system for early tuber development have been presented by Hannapel [34]. Cuttings for the long-day photoperiod were grown in a growth chamber under 16 hr light with the same light intensity and temperature used for cuttings for short-day photoperiod to induce shoot development from axillary buds. Axillary buds were collected directly from either the whole short-day or long-day treated plants at this time and were designated as 0 day buds. Induced axillary buds were harvested by excising the buds from the petiole-leaf cuttings at 4 and 8 days after the beginning of each culture. Mature tubers, stems, leaves, flower, and roots were collected from plants grown under a long-day photoperiod. Unswollen stolons, swollen stolons, and new tubers (characterized by a 2-3 mm diameter swelling at the tip of stolon) were harvested from whole

plants grown under a short-day photoperiod for 4 to 5 weeks after planting. All samples were frozen in liquid nitrogen immediately after harvest and stored at -80°C.

Preparation of MADS-box gene specific probe using PCR

Two primers, an upper primer (5'-CGGAATTCATGGGAAGAGG-3') containing an *Eco* RI site (underlined) and a lower primer (5'-TAAAGCTTACTTCAGCATCACAAAGAACAG-3') containing a *Hind* III site (underlined), were designed based on 30 different flower specific MADS-box DNA sequences selected from GenBank and EMBL databases and were synthesized at the DNA Synthesis Facility at Iowa State University. Template DNAs were prepared from a mass *in vivo* excision of a 4-day axillary bud tuber λZAP®II cDNA library (Stratagene, La Jolla, CA). PCR amplification was carried out in 50 μl reaction mixture that contained 50 pmol of each primer, 400 ng of *Kpn* I linearized early tuber λZAP®II phagemid cDNAs, 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 x PCR buffer A, and 2.0 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). The reaction mixtures were subjected to 40 cycles in a thermal cycler (Perkin-Elmer, Norwalk, CT) using the following sequence: 94°C for 40 seconds, 58°C for 35 seconds, and 72°C for 20 seconds. PCR products were subjected to 1.5% agarose gel electrophoresis and the amplified DNAs were isolated and purified from the agarose gel using the QIAEX® DNA gel extraction system (Qiagen Inc., Chatsworth, CA). A second PCR amplification was performed under the same conditions using the gel-purified PCR product as template.

cDNA library construction and screening

A 4-day axillary bud tuber λZAP®II cDNA library (Stratagene, La Jolla, CA) was constructed according to the manufacturer's protocols. The second PCR product (130 bp in length) labeled with ^{32}P-dCTP using nick translation was used as a probe to screen MADS-box cDNAs from the 4-day axillary bud tuber λZAP®II cDNA library. Plaque lift and phagemid *in vivo* excision were carried out according to the manufacturer's protocols (Stratagene, La Jolla, CA). Plaque hybridization was performed overnight at 42°C in a solution containing 50% formamide, 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 3.3 x Denhardt's solution (1 x Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 25 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0, 0.1mg/ml sonicated salmon sperm DNA, and 0.4% (w/v) SDS. Filters were washed twice in 2 x SSC, 0.1% SDS, for 5 min at room temperature, once in 0.2 x SSC, 0.1% SDS, for 10 min at room temperature, twice in 0.1 x SSC, 0.1% SDS, for 20 min at room temperature, and once in 0.1 x SSC, 0.1% SDS, for 5 min at 61°C, then exposed at -80°C for 18 h to X-ray film for autoradiography. After the third screening, the λ clones were subjected to *in vivo* excision. The pBluescript phagemids excised from the λ clones were purified and evaluated by restriction enzyme digestion analysis. *POTM1*-1 from the pSK102 group [32] was selected for further characterization.

DNA sequencing and computer analysis

Double-stranded DNA sequencing was performed by the dideoxy-nucleotide chain termination method [35] using manual and automated dideoxy sequencing at the Iowa State University DNA Sequencing Facilitity. DNA and deduced amino acids were analyzed using the programs in the GCG software package (University of Wisconsin Genetics Computer Group, Inc., Madison, WI, USA).

Genomic Southern blot analysis

Genomic DNA was extracted from *Solanum tuberosum* L. cv 'Superior' leaves according to the method of Sambrook et al.[36]. Ten μg of DNA was digested to completion with restriction enzymes and subjected to electrophoresis on a 0.8% agarose gel, denatured, and transferred onto nylon membrane (Schleicher and Schuell, Keene, NH) in 25 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0. Prehybridization was at 42°C for 4 to 6 hr in 50% formamide, 6 x SSPE (1 x SSPE is 0.15 M NaCl, 0.25 M NaH_2PO_4, 25 mM Na_2EDTA), 5 x Denhardt's solution, 0.5% SDS, and 0.1 mg/ml sonicated salmon sperm DNA. The membrane was hybridized as described previously [36] with ^{32}P-labeled DNA probes (Fig. 1A) labeled using nick translation. Either the ^{32}P-labeled DNA probe containing the conserved MADS-box sequence (probe 1) or the probe without the conserved MADS-box sequence (probe 2) were used (Fig. 1A and B). The membrane was washed twice for 10 min at room temperature in 2 x SSPE, 0.1% SDS, once for 20 min at room temperature in 1 x SSPE, 0.1% SDS, twice for 20 min at room temperature in 0.1 x SSPE, 0.1% SDS, and once for 5 min at 62°C in 0.1 x SSPE, 0.1% SDS.

Preparation of gene specific RNA probes for northern blot analysis

Two PCR primers were designed and synthesized based on the 3'-UTR of the *POTM1*-1 cDNA. The upper primer (24-mer) begins at nucleotide position 903 and contains an *Eco* RI site (underlined): 5'-CGGAATTCTATCTCTCTCTCTCTC-3'. The lower primer (28-mer) begins at nucleotide position 1044 and contains a *Hind* III site (underlined): 5'-CCAAGCTTAACTGCAAAATCCTTTATTT-3'. PCR amplification was carried out in a 50 μl reaction mixture that contained 30 pmol of each primer, 300 ng of linearized pSK102 plasmid

(*POTM1*-1) as DNA template, 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 x PCR buffer A, and 1.5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). Basic procedures for PCR amplification were the same as previously described for the MADS-box probe. The second PCR products synthesized off the first amplified fragments as a template were cloned into the pCR™II vector (Invitrogen, San Diego, CA) and designated pPR2 (Fig. 1A). PCR products of the MADS-box were cloned into the pCR™II vector and designated pPR1 (Fig. 1A).

Plasmid pPR1 was linearized using *Bam* HI and an antisense MADS-box RNA probe (130 bp) was generated with T7 RNA polymerase (Promega Co., Madison, WI). Plasmid pPR2 was linearized using *Eco* RV and an antisense *POTM1*-1 gene-specific RNA probe (170 bp) was generated with SP6 RNA polymerase (Promega Co., Madison, WI). A linearized oat actin cDNA clone, pOA24 [37], was used to generate an antisense actin RNA probe for a loading control. All the RNA probes were synthesized and labeled with ^{32}P-UTP as described by the procedures of the Riboprobe® System (Promega Co., Madison, WI).

Poly (A)$^+$ mRNA isolation and gel blot analysis

Total RNA was extracted from frozen samples using the phenol/chloroform extraction method [38]. Poly(A)$^+$ mRNA was isolated using an oligo d(T) cellulose column (Collaborative Biomedical Products, Bedford, MA) according to the manufacturer's specifications. Five micrograms of poly(A)$^+$ mRNA were separated electrophoretically on 1.4% denaturing agarose gels using 5mM methyl mercury hydroxide (Alfa Products, Danvers, MA, USA) in 1 x Tris-borate buffer [36]. Each gel was neutralized, stained with ethidium bromide, and blotted onto nylon membranes (Schleicher and Schuell, Keene, NH, USA) in 25 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.0. Membranes were hybridized to RNA probes overnight at 65°C in 50% formamide, 1.0 M NaCl, 10% (w/v) dextran sulfate, 1.0% SDS, and 0.1 mg/ml

salmon sperm DNA. Membranes were washed twice in 2 x SSC, 0.1% SDS, for 5 min at room temperature, once in 0.2 x SSC, 0.1% SDS, for 10 min at room temperature, twice in 0.1 x SSC, 0.1% SDS, for 20 min at room temperature, and once in 0.1 x SSC, 0.1% SDS, for 5 min at 65°C, then exposed for 24 hr to X-ray film for autoradiography. Used membranes were stripped in 0.1 x SSC and 0.1 % SDS at 98°C for 10 min, then exposed to film for 24 hr to confirm the removal of probe. Stripped filters were then hybridized to the antisense actin RNA probe for a loading control.

Results

Isolation of potato MADS box cDNAs and genomic Southern blot analysis

Forty cDNA clones were identified from approximately 7.5 x 10^5 pfu of the potato early tuber cDNA library using the MADS-box probe prepared from PCR amplification. The cDNA structure of *POTM1*-1 is shown schematically in Fig. 1A. The cDNA sequence is 1085 nucleotides in length and contains the MADS-box and the K-box regions. The MADS-box specific probe, pPR1, and the clone-specific probe, pPR2, are indicated (Fig. 1A). Potato (*Solanum tuberosum* L. cv Superior) genomic DNAs digested with *Bam* HI, *Eco* RI, and *Hind* III were hybridized with two DNA probes: probe 1, the full-length cDNA insert and probe 2, without the MADS-box sequence (Fig.1A). As shown in Figure 1B, Southern blot analysis revealed simple banding patterns, suggesting that the *POTM1-1* gene is present in low copy numbers within the genome. Approximately 5.0 and 3.5 kb fragments were detected on *Eco* RI digested genomic fragments using probe 1, but only the 5.0 kb fragment was detected using probe 2, indicating that intervening sequences may have separated the 5'-end of the coding region or that probe 1 has detected another distinct potato MADS-box gene.

Deduced amino acid sequence comparison

From the cDNA sequence, the amino acid sequence of POTM1-1 was deduced and compared to other related MADS-box gene products. *POTM1*-1 cDNA encodes an approximately 29-kD protein of 250 amino acids that contained a 56-residue MADS-box domain and a 53-residue K-box domain. Floral homeotic genes were selected for comparison (Fig. 2) based on sequence homology and their specific functions in flower development. Included in the alignment of Figure 2 are three floral homeotic genes, *AGL3*, *TM3*, and *TobMADS1* [6, 26, 27, respectively] that are also expressed in vegetative organs. Overall, POTM1-1 protein shared 83.2 % homology with TM4 of tomato, 58.8 % with AP1 of *Arabidopsis*, 61.3 % with AGL3 of *Arabidopsis*, 43.6 % TM3 of tomato, 36.3 % with AG of *Arabidopsis*, 38.0 % with TobMADS1 of tobacco, and 33.8 % with DEF A of *Antirrhinum*. The functional MADS-box domain had 100% homology to TM4 and 92.9 % to AP1, but less homology with AGL3 (78 %), AG (73.2 %), TM3 (71.4 %), TobMADS1 (69.6 %), and DEF A (66.1 %). The MADS-box domain of POTM1-1 showed high homology to the invariant residues of SRF-related proteins [4] except for one conservative Lys(K)-to-Arg(R) variation (Fig. 2, residue 5 of POTM1-1). The K-box domain consisted of 53-residues of helix-turn-helix structure [beginning with Glu(E)-94 to Leu(L)-146] in the middle of POTM1-1. POTM1-1's K-box had the highest homology with both TM4 (96.2 %) and AP1 (66.0 %), but lower homology with AG (39.6 %), AGL3 (41.5 %), TM3 (35.8 %), TobMADS1 (35.8 %), and DEF A (30.2 %). Even though, there is considerable diversity in the primary amino acid sequence of the K-box domains, the secondary structure (helix-turn-helix) can be maintained because the consensus hydrophobic amino acid residues [Leu (L), Ile (I), Val (V), and Met (M)] were conserved (Fig. 2).

Within the aligned MADS-box domains, a potential phosphorylation site [Arg(R)-Gln(Q)-Val(V)-Thr(T)] for protein kinases is conserved (Fig. 2). Four putative glycosylation

sites, [Asn(N)-x-Ser(S) and Asn(N)-x-Thr(T)], were identified in POTM1-1, one immediately following the MADS-box domain and three near the carboxy-terminus. The overall alignment showed that the carboxy-terminal region contained more sequence variation than the amino-terminal region. On a sequence homology bases, the alignment showed that POTM1-1 is most closely related to TM4 of tomato and AP1 of *Arabidopsis.* The two proteins of MADS-box genes from solanaceous species that are expressed in both vegetative and floral tissues, TM3, and TobMADS1, showed less sequence homology with POTM1-1, than the floral-specific types, TM4 and AP1. Overall, POTM1-1 has only a 32 % homology to the deduced amino acid sequence of STDEF (33).

Northern blot analysis of POTM1-1 gene expression

To determine the pattern of *POTM1-1* gene expression in different organs, blot hybridization was repeated several times using poly $(A)^+$-enriched mRNA isolated from various potato tissues with the gene-specific antisense RNA probe pPR2 (Fig. 1A). To confirm the specificity of the pPR2 probe, a DNA-DNA cross hybridization analysis was performed using the 170-bp pPR2 gene-specific DNA probe. The results showed that the pPR2 probe did not hybridize to coding sequences from *TM4*, *AG*, or *AP1* (data not shown). These results were reproduced with RNA-DNA hybridization using the pPR2 antisense RNA probe (data not shown), confirming that detectable signals from the pPR2 probe represented *POTM1*-1 transcripts.

To examine *POTM1*-1 gene expression in the potato plant, blot hybridization was performed with poly $(A)^+$-enriched mRNA isolated from various organs. As shown in Figure 3, *POTM1*-1 transcripts, approximately 1.1 kb in length, were detected at the highest levels in leaves (lane LE), with lower levels in roots (lane RT) and vegetative shoot apexes (lane SH), and the lowest levels in stems (lane ST). Unopen and open flowers were used to investigate

early and late stages of flower development, respectively [39]. *POTM1*-1 transcripts were much more abundant in the late flower tissues than in the early flower tissues, indicating that *POTM1-1* gene expression is developmentally regulated during flower development (Fig. 3, lanes EF and LF). To study the expression pattern of *POTM1*-1 during the early stages of tuber development, transcript accumulation was examined during stolon and tuber formation on whole plants. Unswollen stolons (the undifferentiated stage of tuberization), swollen stolons (the initiation stage of tuberization), new tubers (2-3 mm diameter), and mature tubers were collected and used for northern blot analysis (Fig. 4A). As shown in Figure 4B, during stolon and early tuber morphogenesis, *POTM1-1* transcript accumulation was relatively high and constitutive (lanes US, SS, and NT), but significantly reduced in mature tubers (lane MT). Using a detached petiole-leaf cutting system (34), *POTM1*-1 gene expression in leaves was not affected by exogenous treatments of gibberellic acid, benzyladenine, 1-naphthylacetic acid, methyl jasmonate, abscisic acid, and ethylene (data not shown).

Differential expression of POTM1-1 in axillary bud tuber development

A model petiole-leaf cutting system [34] was used to determine the pattern of *POTM1*-1 gene expression during vegetative organ differentiation and development. Clearly distinctive morphological stages were obtained, either microtubers or shoots, during axillary bud development depending on photoperiods (Fig. 5A). Axillary buds of this petiole-leaf cutting system will grow out as tubers if the cuttings are taken from stock plants grown under a short-day photoperiod (8 hr light) or shoots if the stock plants are grown under a long-day photoperiod (16 hr light). Poly $(A)^+$ mRNAs were isolated from axillary buds, axillary bud microtubers, and new shoots harvested either 0, 4, or 8 days after cuttings were taken. *POTM1*-1 transcript levels from the developing shoots from cuttings taken from long-day stock plants increased through 8 days. For the tuberizing axillary buds, the transcript levels

increased up to 4 day, then decreased for 8 day samples (Fig. 5B, SD). *POTM1*-1 transcript levels were approximately equivalent for both 0 and 4 day tissues, regardless of their states of development (tubers or shoots). There is a significant difference, however, between the transcript levels of the 8 day tubers and shoot tissues (compare 8 d lanes in Fig 5B), indicating *POTM1*-1 is differentially expressed during tuber and shoot development depending upon the activity of tissue growth.

Discussion

The Potato MADS-box cDNA designated *POTM1*-1 has been isolated from an early tuber cDNA library using a PCR-amplified MADS-box probe. Southern blot analysis showed that the *POTM1*-1 genomic DNA sequences showed simple banding patterns (Fig. 1B), indicating that low copy numbers of *POTM1*-1 genes are present in the genome. The deduced amino acid sequence of POTM1-1 showed typical features of a MADS-box protein: 56 amino acids of the DNA-binding domain and 53 amino acids of the K-box domain. Comparison with several other MADS-box gene proteins selected on the basis of amino acid sequence and expression similarity showed that the DNA-binding domain of POTM1-1 had very high homology with TM4 (100 %) and AP1 (92.9 %). The invariant residues of the SRF-related proteins were conserved in the amino-terminus of POTM1-1's DNA-binding domain, indicating that the MADS-box domain of POTM1-1 contains specific amino acid sequences involved in DNA-binding [10]. Substantial evidence exists supporting the premise that MADS-box proteins function as transcription factors [1, 12, 14, 40, 41]. Because of the high homology of the MADS-box domain of POTM1-1 to other homeotic proteins, it is very likely that POTM1-1 also functions as a transcription factor in potato. With the exception of TM4, the amino acid sequence of the K-box domain in POTM1-1 has low homology to other MADS-box proteins including STDEF. Despite these differences, the secondary structure of POTM1-1 and the

others is remarkably similar in their helix-turn-helix structure (data not shown). The conserved amino acids beginning with the M-122 to I-139 within the second α-helix in the K-box of POTM1-1 will form an amphipathic α-helix like that in the K-box of TM4. The postulated K-box domain-mediated protein-protein interactions of MADS-box proteins have not been confirmed, but mutation analyses indirectly support the K-box domain's functional role in protein-protein interactions [6, 13, 19, 42]. *BobCAL* from the cultivated garden variety of cauliflower (*Brassica oleracea* var. *botrytis*) [19] and a temperature sensitive mutant *ap3-1* of *Arabidopsis* [7] produced abnormal flower development caused by truncated K-box domains.

As shown in Figure 2, one potential phosphorylation site and four glycosylation sites were contained in POTM1-1. It has been suggested that phosphorylation may be involved in the processing of active MADS-box proteins because the consensus RQVT phosphorylation site is conserved in most MADS-box proteins [3, 5, 26, 43]. Prywes et al. [44] found that phosphorylation of SRF is required to increase DNA-binding activity of SRF to the serum response element (SRE). It is plausible that activation of POTM1-1 is mediated by phosphorylation by specific protein kinases. Glycosylation is also important in the activation of eukaryotic RNA polymerase II transcription factors [45]. POTM1-1 contained four potential glycosylation sites (N-x-S and N-x-T) (Fig. 2). In addition, two potential glycosylation sites in the carboxy-terminus were not conserved between POTM1-1 and TM4, indicating that post-translational modification may distinguish these two similar MADS-box proteins.

The steady state levels of *POTM1*-1 transcripts were high in leaves, shoot apices, roots, stolons, newly formed tubers, but relatively low in stems and mature tubers, suggesting that *POTM1*-1 expression was correlated with active growth. The ubiquitous distribution of *POTM1*-1 transcripts demonstrated a distinctive pattern of expression when compared to other homeotic genes. *POTM1*-1 transcripts were not only abundant in vegetative organs but also in late flowers (Fig 3). Lower levels of transcripts were detected in early flowers compared to late flowers, suggesting that *POTM1*-1 is differentially regulated during flower development.

To further characterize *POTM1*-1 expression during vegetative organ morphogenesis, a model petiole-leaf cutting system was used (Fig. 5). The level of *POTM1*-1 transcripts was essentially the same in the zero day axillary buds collected from whole plants grown under either short-day or long-day conditions. However, *POTM1*-1 transcript levels increased in the 4-day samples of both treatments and in the 8-day shoots from cuttings taken from long-day plants. There was a substantial difference in the levels of *POTM1*-1 transcripts accumulating in 8-day shoots and microtubers (lane 8d in Fig. 5B). There are significant morphological differences between these two samples that might explain this discrepancy: 8-day shoots represent a very active stage of growth with active cell division and expansion; while 8-day microtubers have reached a stage of determinate growth with reduced cell division [29]. The data from the northern blot analysis supports the premise that *POTM1*-1 gene expression is correlated with actively growing tissues.

Overall amino acid sequence comparison indicated that *POTM1*-1 gene is the potato homolog of *AP1*, *SQUAMOS* (the *Antirrhinum* homolog of *AP1*), and *TM4*. These three are floral-specific and are required for floral meristem development as well as sepal and petal development [3, 5, 26, 43, 46]. However, the pattern of *POTM1*-1 gene expression more closely aligns with the vegetative-floral types such as *TobMADS1* cDNA, *TM3* , and *AGL3*. Therefore, in this case, sequence homology does not correlate with pattern of expression. *POTM1*-1's ubiquitous pattern of expression implies that it may be involved in a wider range of morphological events than the floral-specific MADS-box genes. Among the vegetative-floral homeotic genes, *POTM1*-1's distinct amino acid sequence suggests that it represents a unique gene family in this group, functioning as a transcription factor that controls plant growth processes in a number of tissue types.

Acknowledgments

We thank Mary Tymeson, Jennifer K. Hart, Tsu-Hwie Annie Liu, and Eunkyung Lee for their technical support and helpful suggestions. We thank Drs. Martin H. Spalding, Christopher K. Tuggle, James T. Colbert, and Loren C. Stephens for helpful criticism of the manuscript. We thank Dr. James T. Colbert for the pOA24 clone, Dr. E. Lifschitz for the TM4 clone, and Dr. M. Yanofsky for the AG clone. This work was supported in part by the Iowa State University Biotechnology Council. Journal Paper No._____ of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project No. 3056.

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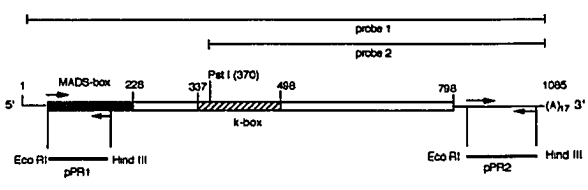
Fig. 1. Schematic diagram of the *POTM1*-1 cDNA and genomic Southern blot analysis. A. *POTM1*-1 cDNA map: The translated region of the *POTM1*-1 cDNA is shown as a box with the DNA sequence encoding a MADS-box shown as solid box and the DNA sequence encoding a K-box shown as a striped box. The lines above the sequence indicate the ^{32}P-labeled DNA probes 1 and 2 used for genomic Southern blot analysis. Two pairs of oligo primers were used for PCR amplification and are indicated by arrows. PCR amplified fragments were cloned into the pCR™II vector (Invitrogen, San Diego, CA) and designated pPR1 and pPR2. The gel-purified *Eco* RI/*Hind* III fragment of pPR1 was used for screening MADS-box cDNAs from the cDNA library. Linearized pPR2 was used to generated ^{32}P-labeled gene-specific RNA probe for northern blot analysis. B. Southern blot analysis with either the ^{32}P-labeled DNA probe containing the conserved MADS-box sequence (probe 1) or without the conserved MADS-box sequence (probe 2). Restriction enzymes used to digest genomic DNA (10 μg) from *Solanum tuberosum* L. cv. Superior leaves are indicated above each lane. B: *Bam* HI, E: *Eco* RI, and H: *Hind* III. Size markers (kb) are indicated in the far right lane.

A.


probe 1
probe 2
1
MADS-box
228
337
Pst I (370)
498
798
1085
5'
(A)17 3'
K-box
Eco RI
Hind III
pPR1
Eco RI
Hind III
pPR2

B.

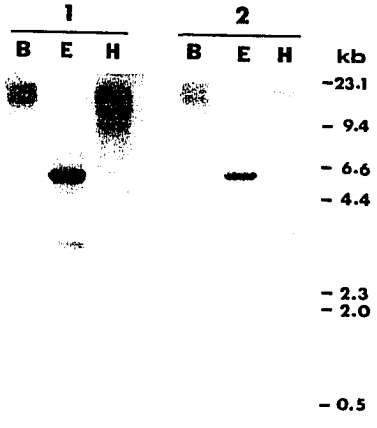
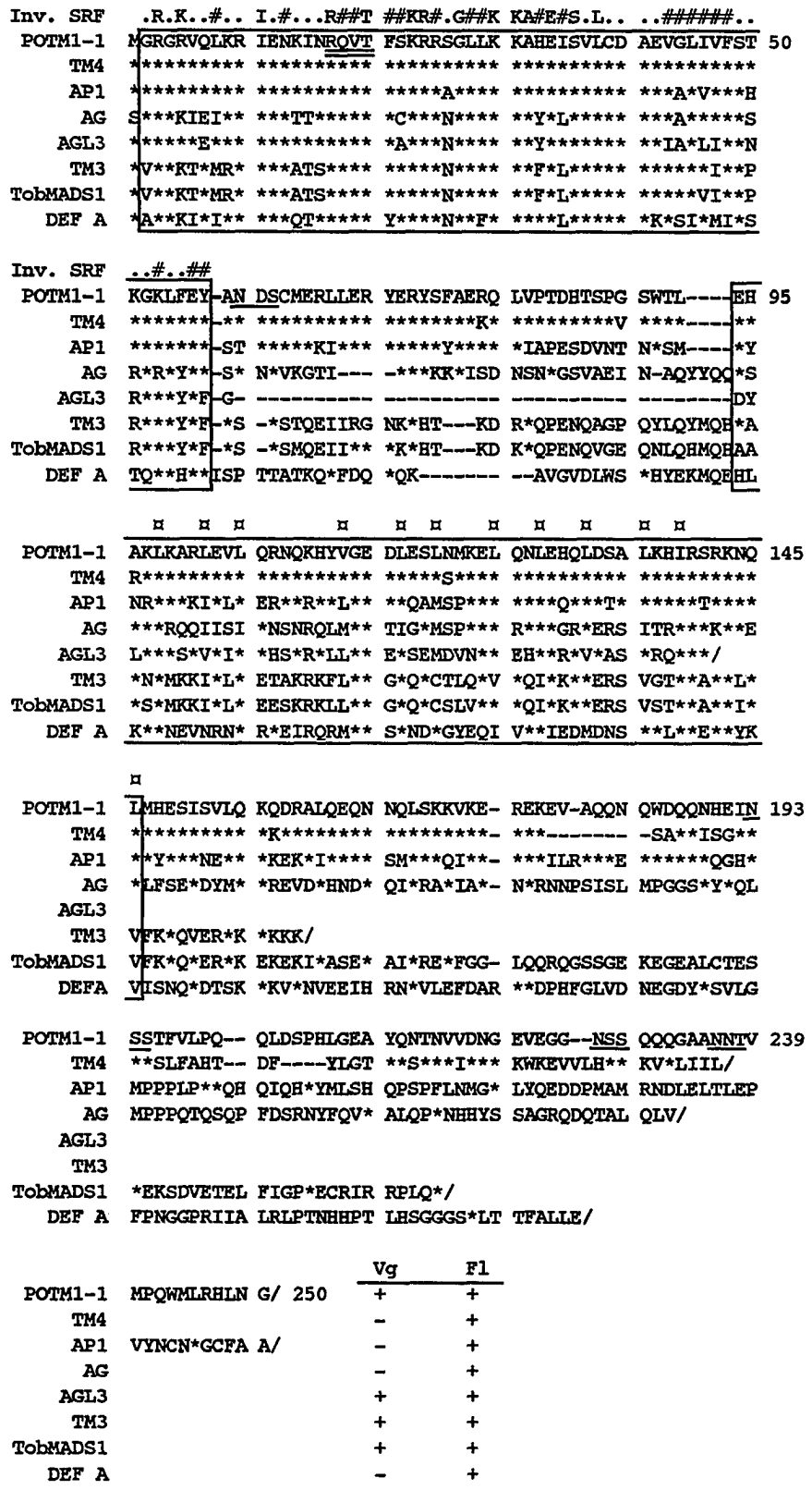

1
2
B
E
H
B
E
H
kb
-23.1
- 9.4
- 6.6
- 4.4
- 2.3
- 2.0
- 0.5

Fig. 2. Deduced amino acid sequence comparison between POTM1-1 and selected MADS-box proteins. The deduced amino acid sequences of TM4 [26], AGL3 [6], AP1 [5], AG [4], TM3 [26], TobMADS1 [27], and DEF A [2] were compared using the Pileup and Gap programs in the GCG software package (University of Wisconsin, Genetics Computer Group, Inc., Madison, WI). The box in the amino-terminus indicates the MADS-box domain. Invariant amino acids of the DNA-binding domain of SRF-related proteins are aligned above the MADS-box domain, together with highly conserved residues indicated by the number (#) symbol and variant residues marked by dots (.). The second box (residues 94—146) of POTM1-1 represents the K-box domain. The conserved hydrophobic residues (L, I, V, M) in the K-box domain are indicated by a symbol (¤) above the K-box domain. Asterisks (*) indicate identical amino acids with POTM1-1; dashes (-) indicate gaps inserted to maximize alignment; slashes (/) indicate the end of each protein. A putative phosphorylation site (RQVT) is double underlined in the MADS-box. Putative glycosylation sites are single underlined. The expression pattern of each gene in vegetative (Vg) and floral (Fl) tissues is shown at the end of the alignment: plus (+) signs represent the presence of transcripts; minus (-) signs represent no transcripts detected.


Inv. SRF .R.K..#.. I.#...R##T ##KR#.G##K KA#E#S.L.. ..######..
POTM1-1 MGRGRVQLKR IENKINRQVT FSKRRSGLLK KAHEISVLCD AEVGLIVFST 50
TM4 ********** ********** ********** ********** **********
AP1 ********** ********** *****A**** ********** ***A*V***H
AG S***KIEI** ***TT***** *C***N**** **Y*L***** ***A*****S
AGL3 ******E*** ********** *A***N**** **Y******* **IA*LI**N
TM3 *V**KT*MR* ***ATS**** *****N**** **F*L***** ******I**P
TobMADS1 *V**KT*MR* ***ATS**** *****N**** **F*L***** *****VI**P
DEF A *A**KI*I** ***QT***** Y****N**F* ****L***** *K*SI*MI*S
Inv. SRF ..#..##
POTM1-1 KGKLFEY-AN DSCMERLLER YERYSFAERQ LVPTDHTSPG SWTL----EH 95
TM4 *******-** ********** ********K* *********V ****----**
AP1 *******-ST *****KI*** *****Y**** *IAPESDVNT N*SM----*Y
AG R*R*Y**-S* N*VKGTI--- -***KK*ISD NSN*GSVAEI N-AQYYQQ*S
AGL3 R***Y*F-G- ---------- ---------- ---------- --------DY
TM3 R***Y*F-*S -*STQEIIRG NK*HT---KD R*QPENQAGP QYLQYMQH*A
TobMADS1 R***Y*F-*S -*SMQEII** *K*HT---KD K*QPENQVGE QNLQHMQHAA
DEF A TQ**H**ISP TTATKQ*FDQ *QK-------- --AVGVDLWS *HYEKMQEHL
¤ ¤ ¤ ¤ ¤ ¤ ¤ ¤ ¤ ¤ ¤
POTM1-1 AKLKARLEVL QRNQKHYVGE DLESLNMKEL QNLEHQLDSA LKHIRSRKNQ 145
TM4 R********* ********** *****S**** ********** **********
AP1 NR***KI*L* ER**R**L** **QAMSP*** ****Q***T* *****T****
AG ***RQQIISI *NSNRQLM** TIG*MSP*** R***GR*ERS ITR***K**E
AGL3 L***S*V*I* *HS*R*LL** E*SEMDVN** EH**R*V*AS *RQ***/
TM3 *N*MKKI*L* ETAKRKFL** G*Q*CTLQ*V *QI*K**ERS VGT**A**L*
TobMADS1 *S*MKKI*L* EESKRKLL** G*Q*CSLV** *QI*K**ERS VST**A**I*
DEF A K**NEVNRN* R*EIRQRM** S*ND*GYEQI V**IEDMDNS **L**E**YK
¤
POTM1-1 LMHESISVLQ KQDRALQEQN NQLSKKVKE- REKEV-AQQN QWDQQNHEIN 193
TM4 ********** *K******** *********- ***-------- -SA**ISG**
AP1 **Y***NE** *KEK*I**** SM***QI**- ***ILR***E ******QGH*
AG *LFSE*DYM* *REVD*HND* QI*RA*IA*- N*RNNPSISL MPGGS*Y*QL
AGL3
TM3 VFK*QVER*K *KKK/
TobMADS1 VFK*Q*ER*K EKEKI*ASE* AI*RE*FGG- LQQRQGSSGE KEGEALCTES
DEFA VISNQ*DTSK *KV*NVEEIH RN*VLEFDAR **DPHFGLVD NEGDY*SVLG
POTM1-1 SSTFVLPQ-- QLDSPHLGEA YQNTNVVDNG EVEGG--NSS QQQGAANNTV 239
TM4 **SLFAHT-- DF-----YLGT **S***I*** KWKEVVLH** KV*LIIL/
AP1 MPPPLP**QH QIQH*YMLSH QPSPFLNMG* LYQEDDPMAM RNDLELTLEP
AG MPPPQTQSQP FDSRNYFQV* ALQP*NHHYS SAGRQDQTAL QLV/
AGL3
TM3
TobMADS1 *EKSDVETEL FIGP*ECRIR RPLQ*/
DEF A FPNGGPRIIA LRLPTNHHPT LHSGGGS*LT TFALLE/
Vg Fl
POTM1-1 MPQWMLRHLN G/ 250 + +
TM4 - +
AP1 VYNCN*GCFA A/ - +
AG - +
AGL3 + +
TM3 + +
TobMADS1 + +
DEF A - +

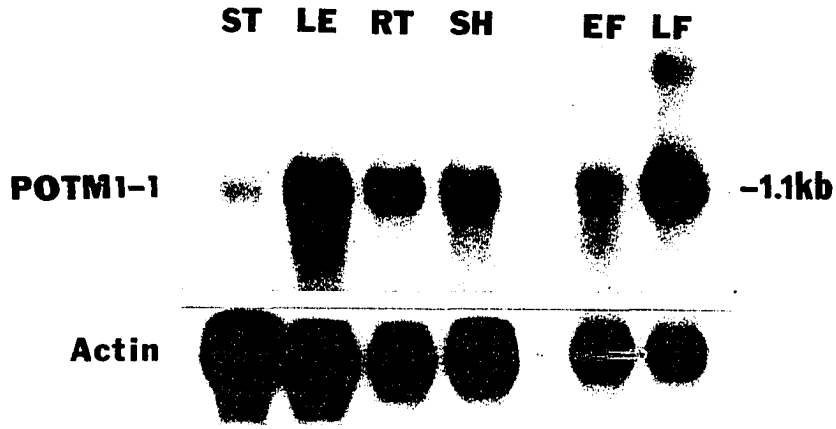


Fig. 3. Northern blot analysis of poly $(A)^+$ mRNA extracted from whole plant organs. Lanes ST, LE, RT, SH, EF, and LF represent, respectively, stems, leaves, roots, shoot tips, early flowers (unopen flower), and late flowers (open flower) of potato cv. Superior. Filters were hybridized with [^{32}P-α-UTP]-labeled *POTM1*-specific antisense RNA probe (170 bp 3'-UTR region of Fig. 1A). Five μg of poly (A^+) mRNA were loaded in each lane. An oat actin RNA probe was used as a control for loading.

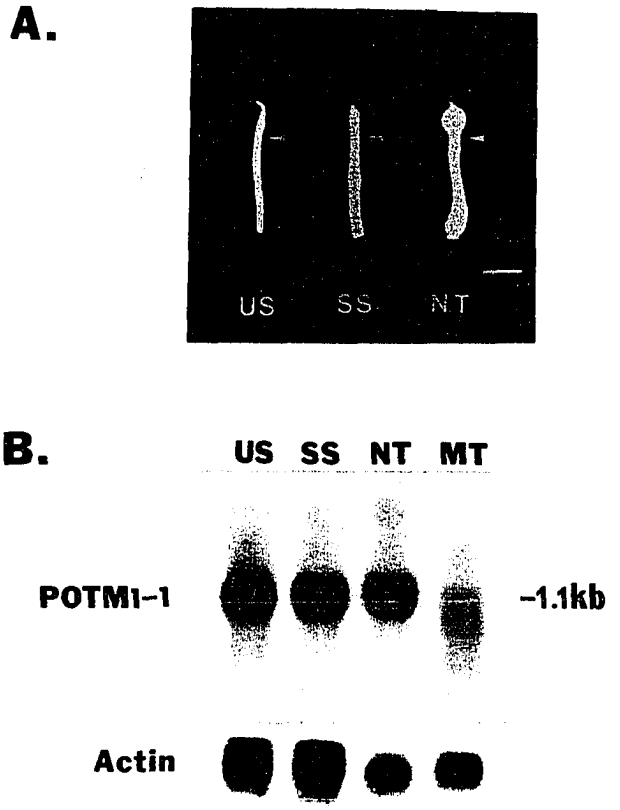


Fig. 4. Northern blot analysis of developing underground stolons from whole plants of the potato cv. Superior grown under a short-day photoperiod. A. Morphology of underground stolon development. US, SS, and NT represent unswollen stolons, swollen stolons, and new tubers, respectively. Stolon tip tissues were harvested above arrows. The bar represents 0.5 cm. B. Northern blot analysis of poly (A^+) mRNA extracted from stolons tip tissues and mature tubers (MT). Filters were hybridized with [^{32}P-α-UTP]-labeled *POTM1*-specific antisense RNA probe (170 bp 3'-UTR region). Five µg of poly (A^+) mRNA were loaded in each lane. An oat actin RNA probe was used as a control for loading.

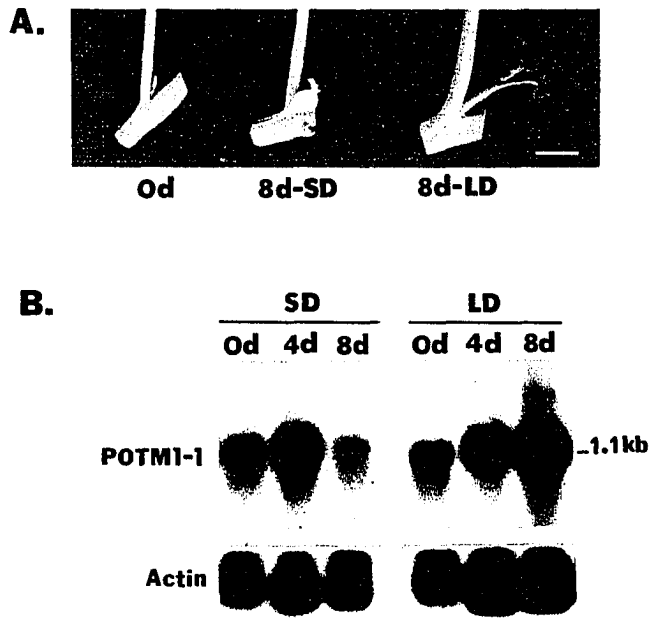


Fig. 5. Northern blot analysis of tuber and shoot tissue from an axillary bud petiole-leaf cutting system from the potato cv. Superior. A. Morphology of petiole-leaf cuttings with the axillary bud intact taken from stock plants grown under either a short-day (8 hr light) or long-day (16 hr light) photoperiod for 3 weeks. The cuttings were cultured in a perlite/vermiculite mix under an 8 hr light (SD) or 16 hr light (LD) photoperiod. After 8 days, the axillary buds from cuttings taken from SD plants grow out as tubers (8d-SD) and those from LD plants grow out as shoots (8d-LD). Zero day buds (0d) were excised directly from the stock plant. The bar represents 1.0 cm. B. Northern blot analysis of poly $(A)^+$ mRNA extracted from 0d axillary buds and 4 and 8d axillary bud tubers (SD) and shoots (LD) excised from petiole-leaf cuttings. Filters were hybridized with [^{32}P-α-UTP]-labeled *POTM1*-specific antisense RNA probe (170 bp 3'-UTR region). Five μg of poly (A^+) mRNA were loaded in each lane. An oat actin RNA probe was used as a control for loading.

CHAPTER 6. GENERAL SUMMARY

To date, little is known about genes involved in vegetative tissue development of potato (*Solanum tuberosum*). The major objectives of this research were to isolate and characterize genes involved in potato vegetative tissue development, including early tuberization. The identification and characterization of genes expressed either specifically or nonspecifically during vegetative growth and early tuberization would be valuable information for further investigation of plant development in general.

In the first part of the research, many cDNA clones were identified from a 4-day axillary bud tuber library using the differential screening technique. One cDNA clone was ultimately chosen to be characterized because it showed low levels of transcripts in stems and mature tubers and high levels of transcripts in early tuber development. DNA sequence analysis revealed that this cDNA was chloroplast (cp) *rps*16 cDNA, which encodes the chloroplast ribosomal protein S16. The coding sequence contained an 88 amino acid open reading frame (ORF) that was interrupted by an 855 bp intron. The deduced amino acid sequence of the cp *rps16* showed 33% homology to the *E. coli* ribosomal protein S16 (Byström et al., 1983) and 95% homology to the tobacco chloroplast ribosomal protein S16 (Shinozaki et al., 1986). The 13 amino acids encoded by exon-1 were highly conserved (100% identity) among higher plants. Two sizes of cp *rps16* transcript were identified from northern blot analysis. Using an intron specific probe, it was shown that the 1.5 kb transcript contained the 855 bp intron and represented an unprocessed RNA molecule, whereas the 0.7 kb transcript represented the mature form. The potato cp *rps16* gene appeared to be constitutively expressed in unswollen stolons and axillary buds, and its expression was enhanced by light. The expression of *rps16* was developmentally regulated, with transcript levels decreasing during tuber development and increasing during shoot development.

It is known that homeotic genes are involved in regulating developmental processes in both animals and plants. Recently, many floral homeotic genes have been isolated and their genetic functions in flower development characterized. Among floral homeotic genes, a family of genes designated the MADS-box genes (Schwarz-Sommer et al., 1990), shares a highly conserved DNA-binding domain (MADS-box) on their deduced amino acid sequences. Although most MADS-box genes of plants function as transcription factors to regulate floral organ development, it is likely that some MADS-box genes may be involved in regulating gene expression that leads to vegetative organ development (Saedler and Huijer, 1993; Weigel and Meyerowitz, 1994). Based on this assumption, the second objective of this research was to identify potato vegetative MADS-gene cDNAs. Using various PCR techniques, several MADS-box gene cDNAs from an early tuber cDNA library were successfully isolated.

From approximately 40 cDNA clones isolated from a 4-day axillary bud tuber cDNA library using a PCR-amplified MADS-box probe, two different groups of clones were identified; the pSK102 group that contained an internal *Pst*I restriction enzyme site and the pSK043 group that did not contain the *Pst*I site. One clone from each group was sequenced and characterized. DNA sequence analysis revealed that both cDNAs were apparently full-length and 99 % identical. The largest ORF of each cDNA encoded the same deduced 250 amino acids (approximately 29 kD) and contained 56 amino acids of the MADS-box domain in the N-terminal region and 53 amino acids of the K-box domain in the middle region. These two cDNAs likely represented different alleles of a potato MADS-box gene (named *POTM1* from this research), designated *POTM1*-1 from the pSK102 group and *POTM1*-2 from the pSK043 group. *POTM1*-1 was 1,085 base pairs (bp) in length and included a 57 bp 5'-untranslated region (UTR), a 750 bp coding sequence (CDS), and a 278 bp 3'-UTR. A typical eukaryotic poly $(A)^+$ signal 5'-AATAAA was located in the 3'-UTR and was followed by a 17 bp poly $(A)^+$ tail. Microsatellite-like AT and CT dinucleotide repeats were located in the 3'-UTR. There are no reports of these short repeat sequences occurring in other homeotic genes.

POTM1-2 is similar to *POTM1*-1, except that it has a 14 bp insertion in the AT repeat sequence, no *Pst*I site, and 6 mismatches in the entire sequence compared to *POTM1*-1.

Southern blot analysis showed that the *POTM1*-1 genomic DNA sequences had simple banding patterns, indicating that low copy numbers of *POTM1*-1 genes were present in the genome. The MADS-box domain of POTM1-1 shared high homology with those of the flower-specific MADS-box proteins, TM4 (Pnueli et al., 1991) of tomato (100 % identity) and AP1 (Mandel et al., 1992b) of *Arabidopsis* (92.9 %). The invariant residues of the SRF-related proteins were conserved in the amino-terminus of POTM1-1's DNA-binding domain, indicating that the MADS-box domain of POTM1-1 contained specific amino acid sequences involved in DNA-binding. The K-box domain of POTM1-1 shared high homology with that of TM4 (96.2 % identity) (Pnueli et al., 1991) but low homology with that of other MADS-box proteins. However, the putative secondary structure of the K-box domain of POTM1-1 showed an amphipathic helix-turn-helix structure that was remarkably similar to K-box domains in other MADS-box proteins. A potential phosphorylation and four potential glycosylation sites were located in POTM1-1, suggesting that posttranslational modification processes might be involved in the activation and specificity of POTM1-1. Overall amino acid sequence comparison indicated that *POTM1*-1 gene is the potato homolog of floral-specific MADS-box genes, *AP1*, *SQUAMOS* (the *Antirrhinum* homolog of *AP1*), and *TM4*. However, the pattern of *POTM1*-1 gene expression was more closely related with the vegetative-floral types such as *TobMADS1*(Mandel et al., 1994), *TM3*, and *AGL3* (Ma et al., 1991). For example, it was observed that the levels of *POTM1*-1 transcripts were high in leaves, roots, aerial shoot tips, underground stolon tips, newly formed tubers, and open flowers, but relatively low in stems, unopen flowers, and mature tubers. In the axillary bud petiole-leaf cutting system, the levels of *POTM1*-1 transcripts increased during the shoot development, but decreased with increasing tuber development. These observations suggest

that *POTM1*-1 gene expression is correlated with morphological development in metabolically active tissues and organs.

In summary, several cDNAs clones were identified and characterized during vegetative organ development and early tuberization of potato. Expression of the potato cp *rps*16 gene was correlated with development of plastids, depending upon cell type and the degree of tissue development. *POTM1*-1 encoded a putative transcription factor containing a MADS-box domain and a K-box domain. The pattern of *POTM1*-1 gene expression was unique from other floral MADS-box genes.

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ACKNOWLEDGMENTS

I am greatly appreciate the support that my parents, a brother, and sisters have given for me.

I express my sincere appreciation to my Ph.D. major professor, Dr. David J. Hannapel for his support, encouragement, and guidance. I would like to thank my former major professors Dr. Han Jip Kim at Ajou University, Sweon, Korea, and Dr. Stephen Surzycki and Dr. Robert Togasaki at Indiana University, Bloomington, IN, for teaching me genetics and molecular biology, and for their encouragement.

I would like to thank the members of my committee, Dr. Clinton F. Hodges, Dr. Richard B. Hall, Dr. Martin H. Spalding, and Dr. James T. Colbert for their help and suggestions. I would like to special thank to Dr. Arthur J. McTaggart at Yeongnam University, Korea, for teaching me English.

I would like to thank professors Dr. Won Hak Park, Dr. Mooung Chang, Dr. Chong Won Rhee, Dr. Ki-Tai Kim, Dr. Kyeong Hee Jun, Dr. Jong Wok Lee, and Dr. Sang-Gon Suh at Yeongnam University, Korea and Dr. Marques E. Kehrli Jr., Dr. Loren C. Stephens, Dr. Bill Summers, and Dr. Mon-Lin Kuo at Iowa State University, Ames, IA, for their help and encouragement.

I greatly thank Dr. Gary F. Polking, Mary Tymeson, Tsu-Hwie Annie Liu, Dr. Hoduck Kang, and Juwhan Eom, for their help, encouragement, and friendship. I thank Haa-young Lee, Jung Tae Kim, Dae-Ryong Kim, Evans Sikiny, Jennifer K. Hart, Tony Aiello, Vasantha Rupasinghe, and Michael Kolomiets, for their help, suggestions, encouragement, and friendship.

A special thanks to my wife, Eunkyong Lee, and my son, Jeongmo Kang (Timothy A. Kang) for their love, support, and faith in me.

To become a Biologist.